UNIVERSIDADE DE LISBOA
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DRUG SUSCEPTIBILITY PROFILING, WHOLE-GENOME MUTATIONAL LANDSCAPE AND SELECTIVE PRESSURE FOOTPRINTS

VANESSA CARDOSO PIRES MARTINS CORREIA

Orientadores: Professora Doutora Helena Paula Lopes Henriques Rebelo de Andrade
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Tese especialmente elaborada para obtenção do grau de Doutor em Ciências e Tecnologias da Saúde, especialidade Microbiologia

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PREFACE

The current thesis presents the data obtained during my PhD research project. The project was carried out since late 2007 in the Antiviral Resistance Lab, Research & Development Unit, Infectious Diseases Department, Instituto Nacional de Saúde Doutor Ricardo Jorge (INSA), IP, Lisbon, Portugal, under the supervision of Prof. Doctor Helena Rebelo de Andrade (INSA, IP; Faculdade de Farmácia, Universidade de Lisboa). Prof. Doctor Pedro Simas (Viral Pathogenesis Lab, Instituto de Medicina Molecular) was the co-supervisor at the Faculdade de Medicina, Universidade de Lisboa.

The first part of the project, which concerns antiviral susceptibility testing, was developed in collaboration with Prof. Doctor Maria Zambon and Doctor Angie Lackenby (Respiratory Viruses Unit, Virus Reference Department, Public Health England, Colindale, London, United Kingdom). They not only provided key guidance and support but also receive me in their laboratory at several time periods (September 2007; November 2007; July 2011), for training and genotypic testing of virus isolates for M2 inhibitor susceptibility (pyrosequencing methodology). Still in this first part and in the context of a collaboration established between the laboratory and the Hospital Curry Cabral, Centro Hospitalar de Lisboa Central, EPE, all influenza viruses from the pandemic period onwards tested for antiviral susceptibility were isolated from clinical specimens provided by the medical doctors Madalena Almeida Santos and Maria José Silvestre (Clinical Pathology Service). The third part of the project focused on the selective pressure forces acting on influenza virus neuraminidase gene was carried out in collaboration with Doctor Ana Abecasis (Global Health and Tropical Medicine, Instituto de Higiene e Medicina Tropical, Universidade NOVA de Lisboa), whom provided key guidance and support. The most computationally demanding analyses were also executed at powerful server systems available in her laboratory. My activity as member of the research group led by Prof. Doctor Helena Rebelo de Andrade at the Host-Pathogen Interaction Unit, Instituto de Investigação do Medicamento (iMed.ULisboa), Faculdade de Farmácia, Universidade de Lisboa, since 2012 contributed to this PhD project through sharing of data and knowledge and general support to the work and underlying scientific activity spreading actions.

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List of original publications included in the thesis

Correia V, Santos LA, Gíria M, Santos MM, Rebelo-de-Andrade H, 2015. Influenza A(H1N1)pdm09 resistance and cross-reduced susceptibility to oseltamivir and zanamivir antiviral drugs. J Med Virol 87(1), 45-56. (APPENDIX A)

Gíria M, Rebelo-de-Andrade H, Santos LA, **Correia V**, Pedro S, Santos MM, 2012. Genomic signatures and antiviral drug susceptibility profile of A(H1N1)pdm09. J Clin Virol 53(2), 140-144. (APPENDIX B)

Santos LA, **Correia V**, Giria M, Pedro S, Santos MM, Silvestre MJ, Rebelo-de-Andrade H, 2011. Genetic and Antiviral Drug Susceptibility Profiles of Pandemic A(H1N1)v Influenza Virus Circulating in Portugal. Influenza Other Respi Viruses 5(Suppl 1), 294–300. (APPENDIX C)

Correia V, Rebelo-de-Andrade H, Santos LA, Lackenby A, Zambon M, 2010. Antiviral drug profile of seasonal influenza viruses circulating in Portugal from 2004/2005 to 2008/2009 winter seasons. Antivir Res 86(2), 128-136. (APPENDIX D)

Santos LA, **Correia V**, Pedro S, Alverca E, Santos MM, Silvestre MJ, Rebelo-de-Andrade H. 2010. Caracterização genética da nova variante pandémica do vírus influenza A(H1N1) 2009 em circulação em Portugal: resultados preliminares. RPDI 6(1), 7-13.

Correia V, Santos LA, Rebelo-de-Andrade H. 2009. Emergência de resistência aos antivirais específicos para a gripe em Portugal. RPDI 5(1), 17-24.

Other publications from the author

David S, **Correia V**, Antunes L, Faria R, Ferrão J, Faustino P, Nunes B, Maltez F, Lavinha J, Rebelo-de-Andrade H, 2017. Population genetics of IFITM3 in Portugal and Central Africa reveals a potential modifier of influenza severity. Immunogenetics, in press.

Meijer A, Rebelo-de-Andrade H, **Correia V**, Besselaar T, Drager-Dayal R, Fry A, Gregory V, Gubareva L, Kageyama T, Lackenby A, Lo J, Odagiri T, Pereyaslov D, Siqueira MM, Takashita E, Tashiro M, Wang D, Wong S, Zhang W, Daniels RS, Hurt AC, 2014. Global update on the susceptibility of human influenza viruses to neuraminidase inhibitors, 2012-2013. Antiviral Res 110, 31-41.

ABSTRACT

Antivirals play an important and decisive role in the clinical management of influenza and in the underlying reduction of related morbidity and mortality. The emergence of antiviral resistance, and particularly of transmissible resistance, poses a serious threat to public health as it could render influenza antivirals useless against circulating viruses. This is even more worrying when considering the current paucity of alternative antiviral therapy choices.

This PhD research project aimed at disclosing the susceptibility of human influenza viruses circulating in Portugal to nationally approved antivirals, and at improving the knowledge on the evolutionary dynamics underlying the emergence and/or spread of influenza variants resistant or with decreased susceptibility to neuraminidase inhibitor (NAI) drugs. To this end, the project focused on three main areas: antiviral susceptibility testing; whole-genome sequencing; and selective pressure (SP) footprints on human influenza neuraminidase (NA)(NAI target).

Antiviral susceptibility testing was performed on human influenza viruses circulating in both community and hospital settings from 2004/2005 to 2012/2013, after establishing a technological platform for comprehensive evaluation of virus susceptibility to M2 protein inhibitors and the NAIs oseltamivir (OS) and zanamivir (ZA) (objective 1). Important findings were made on: the circulation of drug-resistant A(H3N2) (M2 inhibitors) and former seasonal A(H1N1) (OS) viruses; the cut-off for potentially clinically relevant sub-populations of drug-resistant virus; a potential novel amino acid substitution conferring slightly decreased susceptibility to ZA (N2 NA) and a novel source for a variant with decreased susceptibility; and, the virus type or subtype specificity of two amino acid substitutions conferring reduced susceptibility to the drug. Overall susceptibility data contributed at a better understanding of the relationship between virus NAI susceptibility phenotype and genotype and of the natural variations in the *in vitro* NAI susceptibility of circulating viruses over time. The emergence of new drift variants (former seasonal A(H1N1), A(H3N2)), the co-circulation of distinct virus lineages (influenza B) and the increase in OS drug use (A(H1N1)pdm09) were found to potentially play a role in this latter.

Influenza viruses exhibiting resistance or decreased susceptibility to OS and/or ZA were further evaluated through whole-genome sequencing to identify and characterize the

amino acid substitutions specific of their genome (objective 2). No genetic support was found for the fitter NA H275Y OS-resistant former seasonal A(H1N1) viruses, but mutations known to or that based on its structural location or functional impact may play a role in the overall viral fitness, were identified in the genome of single or few viruses resistant or with decreased susceptibility to the drug.

Large datasets of full-length NA gene sequences of worldwide circulating viruses were created to estimate the global and site-specific SP acting on influenza NA, particularly on the sites associated with NAI resistance or reduced susceptibility and/or contacting with the drug (objective 3a). Further temporal splitting of NA gene sequences allowed to investigate for the first time the impact of NAI introduction into clinic (1999) and/or its increased use during 2009 A(H1N1) pandemic on the SP acting on NA (objective 3b). Major findings include: the potential role of positive SP (PSP) in the low-level and locally variable spread of NA H275Y OS-resistant A(H1N1)pdm09 viruses that has been observed in the community; a potential risk of spread of a synergistic drug-resistant (H275Y/S247N) or a RI (S247G) variant in A(H1N1)pdm09 subtype and a RI variant (A395E) in B/Victoria lineage (positive diversifying selection); and the potential lack of impact of both NAI introduction into clinic and its increased use during 2009 A(H1N1) pandemic on the global and site-specific SP acting on influenza NA, with the single exception of site 154 of B/YAM-lineage NA (framework active site residue). Overall mapping of site-specific SP across the different NA subtypes or lineages allowed for further identify 7 potential new regions for drug targeting.

This project marked the beginning of influenza antiviral susceptibility testing and monitoring activities in Portugal. It not only established the technological capacity and capability required to perform such activities but also generated comprehensive information on the susceptibility of circulating human influenza viruses, essential to contribute to both global and European influenza surveillance on antiviral susceptibility. The project also contributed at finding potential determinants of viral fitness in the genome of influenza virus resistant or with decreased susceptibility to NAIs, based on its location onto the protein structure; and at elucidating the role of PSP in the evolutionary pathways to NAI resistance or reduced susceptibility.

Keywords: Influenza; Antiviral susceptibility; Monitoring; Genome-wide sequencing analysis; Positive selective pressure.

RESUMO

Os antivirais assumem um papel de grande relevo na prevenção e controlo da gripe e consequente redução da morbilidade e mortalidade associadas à infecção e suas complicações. O seu uso constitui o único tratamento específico contra o vírus da gripe e, devido às limitações das vacinas atuais, os antivirais podem constituir uma importante estratégia de primeira linha no combate à gripe. A emergência de estirpes resistentes, particularmente se transmissíveis de pessoa-a-pessoa, representa um grave problema de saúde pública, uma vez que pode tornar os antivirais ineficazes contra os vírus influenza em circulação. O limitado leque de alternativas antivirais atualmente disponíveis torna esta situação ainda mais preocupante.

Este projeto de doutoramento pretendeu responder a várias questões na área da resistência aos antivirais, centradas no perfil de susceptibilidade dos vírus influenza em circulação e nos mecanismos moleculares associados à emergência e/ou disseminação de vírus resistentes ou com susceptibilidade reduzida. Nomeadamente:

- Qual o perfil de susceptibilidade dos vírus influenza em circulação em Portugal aos antivirais aprovados para uso clínico? Qual o cenário a nível nacional?
- Poderão mutações nos diferentes segmentos do genoma estar a contribuir para o *fitness* viral de estirpes resistentes ou com susceptibilidade reduzida aos inibidores da neuraminidase (NAIs)?
- Que pressão seletiva (SP) estará a atuar nos locais da neuraminidase (NA) associados ao desenvolvimento de resistência ou redução da susceptibilidade aos NAIs? E nos locais em contacto com o antiviral (sítio ativo da proteína)?
- Qual o impacto da introdução dos NAIs no mercado e do seu maior uso durante a pandemia de 2009 na SP a atuar no gene da NA?

Para responder a estas questões, foram seguidas três linhas de investigação direcionadas para: a avaliação e monitorização da susceptibilidade aos antivirais; a sequenciação do genoma de estirpes resistentes ou com susceptibilidade reduzida; e, a avaliação da SP a atuar no gene da NA.

Avaliação fenotípica e/ou genotípica da susceptibilidade dos vírus influenza em circulação aos antivirais específicos para a gripe

Este estudo envolveu primeiramente a implementação de uma plataforma tecnológica para avaliação da susceptibilidade aos antivirais licenciados em Portugal para uso clínico - amantadina (inibidor da proteína M2) e oseltamivir (OS) e zanamivir (ZA) (NAIs). A plataforma inclui: uma componente de avaliação genotípica aos inibidores da proteína M2, em que susceptibilidade é avaliada através da pesquisa dos 7 marcadores moleculares de resistência na sequência da proteína M2 do vírus; e três componentes de avaliação aos NAIs – fenotípica (valor de IC_{50}), genotípica (sequenciação do gene da NA e da hemaglutinina (HA) do vírus), e, sempre que possível, clínica. No total foram avaliadas mais de 350 estirpes de vírus influenza A e cerca de 530 e 490 estirpes de vírus influenza A e B, em circulação em Portugal entre os Invernos de 2004/2005 a 2012/2013, para determinação da susceptibilidade aos inibidores da proteína M2 e ao OS e ao ZA, respectivamente. Foi detectada resistência aos inibidores da proteína M2 no subtipo A(H3N2) desde o Inverno de 2005/2006, a uma frequência contínua de 100% excepto em 2006/2007 (~75%) (S31N; S31N+V27A), e no subtipo A(H1N1)pdm09 desde a sua emergência (S31N). Praticamente todas as estirpes analisadas demonstraram ser susceptíveis ao OS e ao ZA, excepto no anterior subtipo A(H1N1) sazonal (doravante designado A(H1N1)), em que aproximadamente 21% das estirpes de 2007/2008 demonstraram ser resistentes ao OS (NA H275Y), aumentando para 100% no Inverno seguinte (2008/2009). No subtipo A(H1N1)pdm09, foi detectado um caso de resistência ao OS sobre pressão seletiva de uso do antiviral em uma doente grávida imunocomprometida (NA H275Y), e foi identificada uma sub-população (<30%) de vírus NA H275Y resistentes num caso de aparente relevância clínica (suspeita de resistência). As mutações NA I222V e D198N (numeração N2) estão na base de algumas das reduções observadas na susceptibilidade fenotípica ao OS e/ou ao ZA, tendo sido a primeira vez que variantes NA I222V foram identificadas em doentes não tratados. A mutação NA D251G poderá estar associada a uma redução de 4 vezes na susceptibilidade ao ZA no subtipo A(H3N2) (potencial nova mutação). Reduções ≤2 e ≤4 vezes na susceptibilidade fenotípica aos NAIs aparentam ser uma característica intrínseca dos vírus influenza A e B, respectivamente, não apresentando qualquer base genética (genótipo). A linha base de susceptibilidade aos NAIs (susceptibilidade natural) variou ao longo do tempo, mas sem nenhuma tendência em particular e essencialmente de um modo diferente para o OS e o ZA. A emergência de novas variantes genéticas (A(H1N1), A(H3N2)), a co-circulação de

diferentes linhagens (influenza B), e o maior uso de OS (A(H1N1)pdm09), poderão estar na origem de algumas das variações observadas.

Identificação de potenciais determinantes de fitness viral no genoma de vírus resistentes ou com susceptibilidade reduzida aos NAIs

Os restantes segmentos do genoma (PB2, PB1, PA, NP, M e NS) de 38 estirpes de vírus influenza resistentes ou com susceptibilidade reduzida ao OS e/ou ZA foram adicionalmente sequenciados. Nenhum dos segmentos terá aparentemente contribuído para o maior *fitness* viral da variante NA H275Y A(H1N1) resistente ao OS, em relação aos vírus homólogos *wild-type* (NA H275), que resultou no seu completo predomínio a nível mundial. No entanto, mutações associadas (PB2 V667I; NS1 D74N) ou que, com base no impacto funcional que têm, podem estar associadas (PB2 T108A, D195N, M535L; NEP T48A) a um aumento do *fitness* viral, foram detectadas no genoma de estirpes resistentes ou com susceptibilidade reduzida. De salientar que a análise do gene da HA na primeira parte deste projeto revelou a presença da mutação N156K associada a um maior *fitness* viral em uma estirpe A(H1N1)pdm09 com susceptibilidade reduzida ao OS e ao ZA.

Estudo da pressão seletiva a atuar no gene da NA

Para este estudo foram construídos *datasets* de sequências do gene da NA de vírus influenza a circular a nível mundial, utilizando todas as sequências potencialmente completas disponíveis nas bases de dados públicas GISAID EpiFlu™ e NCBI Influenza Virus Resource até Abril/Novembro de 2013. Foram ainda utilizadas sequências não publicadas de vírus isolados em Portugal. As sequências foram adicionalmente divididas em diferentes *sub-datasets* temporais de modo a investigar pela primeira vez o impacto da introdução dos NAIs no mercado (1999) e do seu maior uso durante a pandemia de 2009 na SP a atuar no gene da NA (alvo dos NAIs).

No geral, os locais da NA associados ao desenvolvimento de resistência ou redução da susceptibilidade aos NAIs e/ou em contacto com o antiviral encontram-se essencialmente sob SP negativa (NSP) (A(H3N2), linhagem B/Victoria (B/VIC)) ou sob NSP ou uma razão dN/dS<1 não significativa (A(H1N1), linhagem B/Yamagata (B/YAM), A(H1N1)pdm09). No entanto, em todos os subtipos ou linhagens de vírus influenza excepto na linhagem B/YAM foram detectados locais sob SP positiva (PSP), incluindo os sites 275 e 247 da NA dos vírus A(H1N1)pmd09 e o site 395 da NA dos vírus da linhagem B/VIC. A evidência de PSP nestes locais suporta um potencial papel da PSP na disseminação limitada e

geograficamente variável de vírus NA H275Y A(H1N1)pdm09 resistentes ao OS que tem vindo a ser observada na comunidade, entre doentes não tratados; e alerta para um potencial risco de disseminação de uma variante A(H1N1)pdm09 sinergicamente resistente ao OS (H275Y/S247N) ou com susceptibilidade reduzida (S247G) e de uma variante B/VIC com susceptibilidade reduzida (A395E). A PSP detectada no site 275 da NA dos vírus A(H1N1) e nos sites 148 e 151 da NA dos vírus A(H3N2) pode ser somente um artefacto de, respectivamente, a disseminação global de uma estirpe NA H275Y resistente ao OS e o isolamento e propagação em cultura celular. Quer a introdução dos NAIs no mercado, quer o seu maior uso durante a pandemia de 2009, não tiveram aparentemente um impacto na SP a atuar no gene da NA, excepto no site 154 do sítio ativo da NA dos vírus B/VIC, que revelou estar sob uma SP significativamente mais forte durante o período de maior uso antiviral. O mapeamento da SP a atuar sobre todos os locais da NA nos diferentes subtipos ou linhagens de vírus influenza permitiu a identificação de 7 novos potenciais alvos para fármacos antivirais.

Este projeto de doutoramento permitiu dispor da capacidade tecnológica para iniciar a avaliação e monitorização da susceptibilidade dos vírus influenza aos antivirais a nível nacional, resultando numa das maiores coleções de informação sobre susceptibilidade dos vírus influenza em circulação à data (10 anos). Esta informação foi essencial para contribuir para a vigilância realizada na Europa e a nível global e para novos avanços no conhecimento sobre a relação fenótipo-genótipo na susceptibilidade do vírus aos NAIs, a qual constitui um dos maiores desafios na área. Os restantes resultados do projeto contribuíram para esclarecer a presença de potenciais determinantes de *fitness* viral no genoma de vírus resistentes ou com susceptibilidade reduzida e para elucidar o papel da PSP na emergência e/ou disseminação de vírus resistentes ou com susceptibilidade reduzida. Contribuíram ainda para a identificação de potenciais novos alvos antivirais, o que constitui uma das prioridades atuais na área da gripe devido ao limitado repertório de alternativas antivirais disponíveis.

Palavras-chave: Vírus influenza; Susceptibilidade aos antivirais; Monitorização; Sequenciação completa do genoma; Pressão seletiva positiva.

THESIS OUTLINE

This PhD thesis is organized in 6 chapters, which are preceded by an abstract and a summary written in Portuguese. Chapter 1 presents an introductory review of the subject, covering general aspects of influenza virus and influenza NA protein and addressing the most up-to-date literature on influenza antivirals and antiviral resistance. A description of the surveillance monitoring activities for antiviral resistance and their importance close the review. Chapter 2 describes in more detail the problems under investigation and the purpose and significance of this research project, presenting the design of the study and the main approaches used. Project aims and objectives are specified at the end of the chapter. Chapter 3 provides a description of the material and methods used to carry out the project work, while the resulting data is presented and discussed in chapters 4, 5 and 6, with each chapter covering a different objective of the research. The scientific articles published or, when absent, the oral communications presented, and a list of the main activities carried out, are indicated at the beginning of these latter chapters. Major findings are listed at the end, followed by supplementary data. The final and overall conclusions of the research are presented in a final concluding remarks section, in which are also highlighted potential and important directions to take in future follow-ups of these investigations.

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ABBREVIATIONS AND ACRONYMS

AIC	Akaike's information criterion
aLRT	approximate likelihood-ratio test
AMA	amantadine
AMV RT	avian myeloblastosis virus reverse transcriptase
AVWG	Expert Working Group on Surveillance of Influenza Antiviral Susceptibility
BR/07	A/Brisbane/59/2007
B/VIC	B/Victoria
B/YAM	B/Yamagata
CDC	Centers for Disease Control and Prevention
cDNA	complementary DNA
CL	chemiluminescent
CNS	central nervous system
CPE	cytopathic effect
cRNA	complementary RNA
DANA	2-deoxy-2,3-dehydro-N-acetylneuraminic acid
DMEM	Dulbecco's modified Eagle's medium
dN	rate of non-synonymous substitutions
dNTP	deoxynucleoside triphosphate
dS	rate of synonymous substitutions
dsRNA	double-stranded RNA
E'	vestigial esterase
ECACC	European Collection of Authenticated Cell Cultures
ECDC	European Centre for Disease Prevention and Control
EISN	European Influenza Surveillance Network
ERLI-Net	European Reference Laboratory Network for Human Influenza
FBS	fetal bovine serum
FEL	fixed effects likelihood
FL	fluorescent
fw	forward
GISAID	Global Initiative on Sharing All Influenza Data
GISRS	Global Influenza Surveillance and Response System
HA	hemagglutinin
HCC	Hospital Curry Cabral
HPA	Health Protection Agency
HRI	highly reduced inhibition

IC_{50}	concentration of antiviral required to inhibit a standardised amount of virus NA activity by 50%
IFN	interferon
IHMT	Instituto de Higiene e Medicina Tropical
ILI	influenza-like illness
INSA	Instituto Nacional de Saúde Doutor Ricardo Jorge
LAN	laninamivir
LANO	laninamivir octanoate
LE	lower extreme
LM	lower mild
M-MLV RT	Moloney murine leukemia virus reverse transcriptase
M1	matrix protein
MDCK	Madin-Darby canine kidney
MEM	minimum essential medium
min	minutes
ML	maximum-likelihood
mRNA	messenger RNA
MUNANA	2′2′-(4-Methylumbelliferyl)-α-D-N-acetylneuraminic acid sodium salt hydrate
n.s.	not significant
NA	neuraminidase
NAI	neuraminidase inhibitor
NC/99	A/New Caledonia/20/1999
NCBI	National Center for Biotechnology Information
NEAA	non-essential amino acids
NEP	nuclear export protein
NES	nuclear export signal
NI	normal inhibition
NIC	National Influenza Centre
NIMR	National Institute for Medical Research
NISN	Neuraminidase Inhibitor Susceptibility Network
NMR	nuclear magnetic resonance
NNI	nearest-neighbour-interchange
NP	nucleocapsid protein
NS1	non-structural protein 1
NS2	non-structural protein 2
NSP	negative selective pressure
nts	nucleotides
ORF	open reading frame
OS	oseltamivir
OSC	oseltamivir carboxylate

OSP oseltamivir phosphate
PBS phosphate buffered saline
PCR polymerase chain reaction
PDB Protein Data Bank
PER peramivir
PHE Public Health England
PSN penicillin-streptomycin-neomycin antibiotic mixture
R receptor binding
RBCs guinea pig red blood cells
RBS receptor binding site
rev reverse
RFUs relative fluorescence units
RG reverse genetics
RI reduced inhibition
RIM rimantadine
RLUs relative light units
RNP ribonucleoprotein
RT reverse transcription
S_SP stronger selective pressure
S/N signal to noise ratio
SARS severe acute respiratory syndrome
SD standard deviation
SE standard error
sec seconds
SH-like Shimodaira-Hasegawa-like
SIAT1 β-galactoside α2,6-sialyltransferase
SLAC single-likelihood ancestor counting
SMAD scaled median absolute deviation
SNP single nucleotide polymorphism
SP selective pressure
SPR subtree-pruning-and-regrafting
TBE tris-borate-EDTA
TEM transmission electron microscopy
TM transmembrane
UE upper extreme
UM upper mild
UK United Kingdom
USA United States of America
UV ultraviolet
VIRGIL Vigilance Network for the Management of Antiviral Drug Resistance

vRNA	viral RNA
W_SP	weaker selective pressure
WHO	World Health Organization
WHO CCs	WHO Collaborating Centres
ZA	zanamivir

"The ideal scientist thinks like a poet and only later works like a bookkeeper. Keep in mind that innovators in both literature and science are basically dreamers and storytellers. In the early stages of the creation of both literature and science, everything in the mind is a story. There is an imagined ending, and usually an imagined beginning, and a selection of bits and pieces that might fit in between. In works of literature and science alike, any part can be changed, causing a ripple among the other parts, some of which are discarded and new ones added. The surviving fragments are variously joined and separated, and moved about as the story forms. (….) To bring the end safely home is the goal of the creative mind. Whatever that might be, wherever located, however expressed, it begins as a phantom that rises, gains detail, then at the last moment either fades to be replaced, or, like the mythical giant Antaeus touching Mother Earth, gains strength. Inexpressible thoughts throughout flit along the edges. As the best fragments solidify, they are put in place and moved about, and the story grows until it reaches an inspired end."

Edward O. Wilson, Letters to a Young Scientist, 2013

CHAPTER 1

LITERATURE REVIEW

1.1 INFLUENZA VIRUS: GENERAL CONCEPTS

The influenza virus is a complex, constantly changing virus [1]. Notorious by its unique ability to continually evade the host's immune system, it also knows no boundaries, circulating around the world within species and occasionally jumping between them [2]. Its evolutionary pathways are completely unpredictable. From him we can only *"expect the unexpected"* [3].

1.1.1 Classification and Nomenclature

Influenza viruses belong to the family *Orthomyxoviridae* (from the Greek *orthos*, straight; and *myxa*, mucus) that is defined by viruses with a negative-sense, single-stranded and segmented RNA genome [4,5]. Presently, the family includes seven different genera: *Influenzavirus A*; *Influenzavirus B*; *Influenzavirus C*; *Influenzavirus D; Thogotovirus*; *Isavirus*; and *Quaranfilvirus* [5,6]. Influenza viruses are divided into 4 genera (commonly called types). The division into types A, B and C is based on the antigenic differences between their nucleocapsid protein (NP) and matrix protein (M1) [7]. Type D was only recently recognized as a new virus genus, separated from type C, based on overall genetic and antigenic differences evidenced by their highly divergent genomes (≈50%), distinct mechanisms of protein expression (M1), inability to reassort and yield viable progeny and lack of antibody cross-reactivity [8]. Influenza A, B and C viruses are evolutionary related, sharing a common ancestor from which type A and B viruses have diverged more recently, compared to type C [5]. Influenza D virus emerged very recently (2011), being more closely related to type C virus [9].

Influenza A virus infects a variety of hosts, including humans, birds, swine, horses, dogs and, more rarely, others animals such as cats, a variety of sea mammals and camels [10]. Aquatic birds are known to be its natural reservoir and the source of virus in other species [11]. Type B and C viruses have been primarily isolated from humans [10], while influenza D virus primarily affects cattle and is not known to infect humans [6]. Influenza A virus is the most diversified and is further subdivided into subtypes, according to the antigenicity of their hemagglutinin (HA) and neuraminidase (NA) surface proteins. Currently, there are 18 recognized HA subtypes and 11 NA subtypes [12]. Combinations of all these subtypes have been found in viruses of avian species, while only three HA (H1,

H2, H3) and two NA (N1, N2) subtypes have been found as components of human influenza viruses [13]. No antigenic subtypes have been identified among circulating type B, C and D viruses [4,6]. Only type A and B viruses are clinically relevant for humans, causing influenza epidemics. Type C virus causes only mild disease or sub-clinic infection [14]. Both influenza C and D viruses were not studied in this PhD work and therefore will no longer be addressed in this review.

The current nomenclature system for influenza viruses (WHO Memorandum 1980 [15]) includes the genus (type), the species from which the virus was isolated (omitted if human), location of the isolate, number of the isolate and year of isolation. In the case of influenza A virus, it also includes the specific subtype of HA and NA proteins between brackets (e.g. A/swine/Lisbon/44/2001 (H3N2)).

1.1.2 Virion Structure

The infectious virus particle, also called virion, of influenza A and B viruses is very similar. Although being pleomorphic, it usually assumes a roughly spheroidal form (~100nm in diameter), with filamentous forms (1μm or longer) being frequently observed, particularly in fresh clinical isolates [5,16]. The most distinctive feature of influenza A and B virions is a layer of spikes projecting radially from the surface, as clearly evidenced in electron micrographs (Figure 1.1).

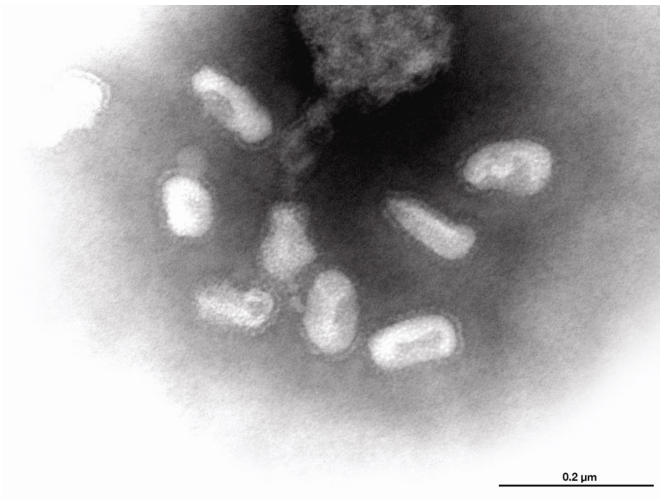

Figure 1.1 Virus particles of the influenza A/Portugal/02/2009 (H1N1pdm09) isolate visualized by transmission electron microscopy (TEM).

(Footnote Figure 1.1)
This TEM image was taken in the context of the study developed by our research team on the newly emergent 2009 pandemic A(H1N1) virus (published in Santos *et al.* [17]). I and another colleague prepared the virus for observation, through negative staining, using 2% ammonium molybdate. A colleague from the laboratory of Biology and Ecotoxicology, Department of Environmental Health, Instituto Nacional de Saúde Doutor Ricardo Jorge, I.P. (Lisbon, Portugal), collected the images of the virus particles on a Morgagni 268D electron microscope (FEI Company, Eindhoven, The Netherlands).

These spike-like structures correspond to HA (rod-shaped spikes) and NA (mushroom-shaped spikes) proteins that project about 10 to 14nm from the virus surface, constituting the major surface antigens of the virus [18]. On spherical-shaped virions there are about 500 HA and 100 NA spikes (HA/NA ratio of 5:1), while those with a filamentous form contain hundreds more. Both HA and NA spikes are distributed around the virus membrane but NA apparently clusters in the region where the budding virus particles are released from cells [13].

Each influenza A or B virion comprises an envelope, a matrix layer and a ribonucleoprotein (RNP) core [16]. The envelope is formed by a lipid bilayer originated from the host cell. Inserted into this lipid membrane are not only the HA and NA antigens, but also the M2 ion channel protein, in the case of influenza A virions, or the NB and BM2 proteins, in the case of influenza B virions [5]. The matrix layer resides underneath the viral membrane and is composed by a single protein – M1. M1 is the most abundant protein of the virion, having the responsibility to provide shape and stability to the lipid envelope [19]. It is further suggested that M1 forms the critical bridge between the viral envelope and the inner core components, by interacting with both the cytoplasmic tails of the membrane proteins and the viral RNA (vRNA) and NP [5]. The virion core contains 8 segments of vRNA (viral genome). Each segment is associated with multiple copies of NP and with the viral RNA-dependent RNA polymerase composed by PB1, PB2 and PA proteins, forming the designated RNP complex [4]. By being wrapped around the NP copies, the vRNA assumes a helical hairpin form that is bound at one end by the heterotrimeric RNA polymerase [5]. The nuclear export protein (NEP), initially designated as non-structural protein 2 (NS2), is also present in the virus core, in small amounts and probably in association with the M1 [20].
Figure 1.2 shows the schematic representation of an influenza A virion.

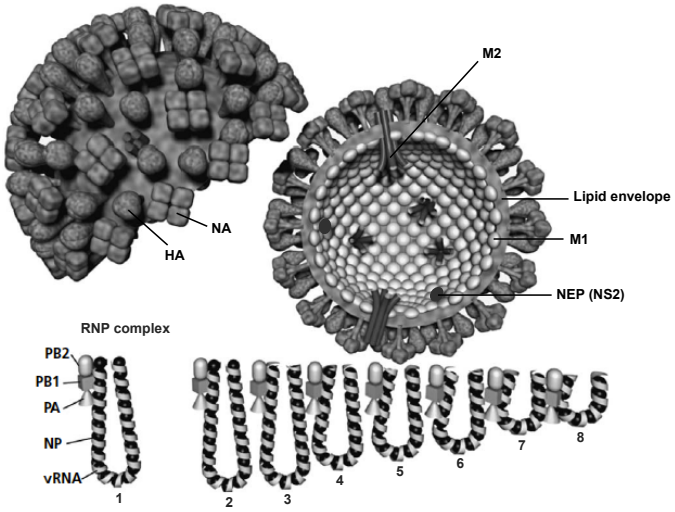

Figure 1.2 Schematic representation of an influenza A virus particle.

The hemagglutinin (HA), neuraminidase (NA), and M2 ion channel proteins are inserted into the host-derived lipid envelope, while the matrix protein (M1) is located underneath, forming a layer. Each of the 8 viral RNA (vRNA) segments is wrapped up around nucleoprotein (NP) and bound by the heterotrimeric RNA polymerase (PA, PB1 and PB2 subunits), forming a ribonucleoprotein (RNP) complex. The position of the nuclear export protein (NEP; also known as non-structural protein 2 (NS2)) in the inner core is not clear but it probably interacts with the M1. Adapted from Cox *et al.* [4].

1.1.3 Genome Organization and Encoded Proteins

The 8 RNA segments that constitute the genome of influenza A and B viruses are numbered in descending order from the longest (segment 1) to the shortest (segment 8). The segments also vary in length according to the influenza type, with all presenting a longer length in influenza type B viruses (Table 1.1). These have, therefore, the longest genome (~14600 nucleotides (nts)), which differs in approximately 1000 nts from the genome of influenza A viruses (~13600 nts). Notwithstanding, the shorter genome of influenza A viruses, particularly the first three segments, encodes a higher number of proteins. Up to date, it was found to encode 15 different proteins, contrasting with the 11 proteins codified by the RNA segments of influenza B virus genome [21,22]. Table 1.1 shows the viral proteins encoded in the different RNA segments of influenza A and B virus genome and describes its main functions.

The three largest vRNA segments encode the three subunits of the viral RNA-dependent RNA polymerase: PB2 (segment 1), PB1 (segment 2), and PA (segment 3) proteins. Segment 2 (also called PB1 segment) encodes two additional proteins in influenza A viruses. The PB1-F2 protein, which is translated in the +1 open reading frame (ORF) of

PB1 [23]; and the PB1-N40 protein that is translated from the fifth initiation codon (located at position +40) in the same ORF as PB1, consisting in a N-terminally truncated variant of this core protein [24]. Segment 3 (or PA segment) of influenza A viruses also encodes additional proteins. Specifically, the PA-X protein, which is encoded by an alternative ORF of PA accessed via ribosomal frameshifting [25]; and PA-155 and PA-N182 proteins, both translated in the same ORF as PA from, respectively, the eleventh (position +155) and thirteenth (position +182) initiation codons, forming N-terminally truncated variants of PA [22]. These three additional proteins, as well as the PB1-F2 and PB1-N40 proteins encoded in PB1 segment, have been detected in influenza A virus-infected cells, but are not known to be incorporated into progeny virions [26].

Genome segments 4, 5 and 6 encode the HA, NP and NA proteins, respectively. In influenza B viruses, segment 6 encodes an additional protein designated NB. This protein is translated from a –1 ORF, starting just 4 nts upstream of the NA coding frame. Up to date, NB has shown to be completely conserved [27].

Segment 7 encodes the M1 and a second protein that differs according to the influenza virus type. In influenza A viruses encodes the M2 protein via alternative mRNA splicing, while in influenza B codes for the BM2 protein through a "stop–start" mechanism, on which the termination codon for M1 overlaps with the initiation codon for BM2 [21].

Segment 8 encodes the non-structural protein 1 (NS1) and, via a spliced mRNA, the NEP (NS2) protein. NS1 has not been detected in the virion, hence its designation as non-structural protein.

Recently discovered influenza A virus PB2-S1 (segment 1), M42 (segment 7) and NS3 (segment 8) proteins were not considered as were either only express in former seasonal A(H1N1) viruses (PB2-S1) or only found in a limited number of influenza A viruses (M42 and NS3) [28-30].

HA and M2 proteins will be described in more detail below given its importance for influenza antiviral susceptibility. The former may be involved in the development of resistance to neuraminidase inhibitor (NAI) drugs, while the latter is the target of the first available class of anti-influenza drugs – M2 protein inhibitors. NA protein constitutes the target of NAI drugs and was widely studied in this PhD work. For this reason, it will have its own section (see section 1.2).

Table 1.1 Viral RNA segments of influenza A and B virus genome and encoded proteins.

Genome segment	Common designation	Lenght (influenza virus type)	ENCODED PROTEINS		
			Designation	Length (influenza virus type)	Function
1	PB2	2341 nts (A); 2369 nts (B)	PB2	759 aa (A); 770 aa (B)	◆ RNA polymerase subunit ◆ Binding the 5´cap on host pre-mRNAs ◆ Required for vRNA replication
2	PB1	2341 nts (A); 2368 nts (B)	PB1	757 aa (A); 752 aa (B)	◆ RNA polymerase subunit ◆ Catalysing the sequential addition of nucleotides during RNA chain elongation ◆ Contains the conserved motifs characteristic of RNA-dependent RNA polymerases ◆ Binding the terminal ends of both vRNA and cRNA to initiate transcription and replication ◆ Structural backbone of RNA polymerase (interacts with both PB2 and PA)
			PB1-F2	87 aa (A) [a]	◆ Virulence factor by inducing mitochondria-associated apoptosis (pro-apoptotic activity) ◆ Pro-inflammatory properties ◆ Interacts with PB1 and influences the viral polymerase activity
			PB1-N40	718 aa (A)	◆ Maintaining the balance between PB1 and PB1-F2 expression
3	PA	2233 nts (A); 2245 nts (B)	PA	716 aa (A); 726 aa (B)	◆ RNA polymerase subunit ◆ Endonuclease involved in generating the capped primers for viral mRNA synthesis (cap-snatching) ◆ Required for vRNA replication ◆ Interacts with PB1 ◆ Proteolytic activity
			PA-X	252 aa (A)	◆ Modulates the host response and viral virulence
			PA-N155	562 aa (A)	◆ Unknown
			PA-N182	535 aa (A)	◆ Unknown
4	HA	1778 nts (A); 1882 nts (B)	HA	566 aa (A)[b]; 584 aa (B)	◆ Major antigenic determinant ◆ Binding the sialic acid moieties on host-cell receptors, promoting virus entry into target cells ◆ Promoting fusion between the virus and endosome membranes to deliver the virus genome into the cell ◆ Putative structural role in virus budding and particle formation
5	NP	1565 nts (A); 1841 nts (B)	NP	498 aa (A); 560 aa (B)	◆ Major component of the viral RNP complex ◆ Mediating the nuclear import of viral RNP complexes ◆ Involved in regulating the switch of RNA synthesis from transcription to replication ◆ Target of the host's cytotoxic T-cell mediated immune response (internal antigen; type-specific)

(Table 1.1 cont.)

Genome segment	Common designation	Lenght (influenza virus type)	ENCODED PROTEINS		
			Designation	Length (influenza virus type)	Function
6	NA	1413 nts (A); 1557 nts (B)	NA	454 aa (A)[b]; 486 aa (B)	◆ Surface antigen ◆ Removal of the sialic acid moieties on host-cell receptors and newly synthesized HA and NA proteins, promoting the release of progeny virions from infected cells ◆ Removal of the sialic acid moieties on the mucus and cell surfaces of the respiratory tract, promoting virus spread ◆ Putative role in assisting virus entry and/or enhancing late endosome/lysosome traficking
			NB	100 aa (B)	◆ Unknown
7	M	1027 nts (A); 1180 nts (B)	M1	252 aa (A); 248 aa (B)	◆ Providing shape and stability to the lipid envelope - matrix protein ◆ Forming a bridge between membrane proteins and the inner core components of the virion apparently essential for virus assembly at the plasma membrane ◆ Involved in the nuclear export of viral RNP complexes
			M2	97 aa (A)	◆ Proton-selective channel ◆ Conducting protons from the acidified endosomes into the interior of the virus to dissociate the RNP complexes from the M1 protein, allowing its release as free structures ready for nuclear import
			BM2	109 aa (B)	◆ Proton-selective channel; functional equivalent of type A M2 protein in influenza B viruses
8	NS	890 nts (A); 1096 nts (B)	NS1	230 aa (A); 281 aa (B)	◆ Suppressing virus-induced host type I IFN-mediated antiviral response - IFN antagonist ◆ Regulating mRNA splicing and the nuclear export of cellular mRNAs to maximize viral mRNA synthesis ◆ Stimulating viral mRNA translation ◆ Inducing apoptosis in virus-infected cells
			NEP (NS2[c])	121 aa (A); 122 aa (B)	◆ Mediating the nuclear export of viral RNP complexes ◆ Involved in regulating RNA synthesis

aa: amino acids; cRNA: complementary RNA; HA: Hemagglutinin; IFN: Interferon; M1: Matrix protein; mRNA: messenger RNA; NA: Neuraminidase; NEP: Nuclear export protein; NP: Nucleocapsid protein; NS1: Non-structural protein 1; NS2: Non-structural protein 2; nts: nucleotides; RNP: Ribonucleoprotein; vRNA: viral RNA

[a] Can be truncated at either the N- or C-terminal ends; [b] The length differ across the different subtypes and may even differ among virus strains; [c] Initial designation

Based on Krug and Fodor [21]; Gastaminza *et al.* [31]; Braam *et al.* [32]; Gonzalez and Ortin [33]; Cox *et al.* [4]; Conenello *et al.* [34]; Vasin *et al.* [35]; Huarte *et al.* [36]; Sanz-Ezquerro *et al.* [37]; Shaw and Palese [5]; Russell *et al.* [13]; Braciale [38]; Wu *et al.* [39]; Wrigley [19]; Nayak *et al.* [40]; Wright *et al.* [10]; Chen *et al.* [23].

1.1.3.1 Hemagglutinin

HA is the major surface protein of influenza A and B viruses. It is a rod-shaped homotrimer of non-covalently linked monomers, with the C-terminus inserted into the viral membrane and the hydrophilic end projecting as a spike away from the viral surface - type I integral membrane protein [4,5]. As formerly indicated in Table 1.1, HA is a major antigenic determinant, inducing the formation of neutralizing antibodies that play an important role in protecting the host against infection [41]. It has two major functions in the virus life cycle, both in early stages: (1) binding the α-2,3 or α-2,6-linked sialic acid moieties on host-cell receptors, enabling the attachment of the viral particle to the host cell (virus attachment); and (2) mediating the fusion between the virus and endosome membranes, allowing the release of viral RNP complexes into the cytoplasm of the host cell (virus uncoating). It has also been suggested that HA may play a structural role in virus budding and particle formation [5].

Each monomer of HA is synthesized as a single polypeptide chain (HA0) that undergoes post-translational modifications, including the cleavage by host-cell proteases into two subunits, HA1 and HA2, linked by a single disulfide bond. HA0 cleavage is triggered by low pH in the endosome and is required for infectivity to expose the hydrophobic N-terminus of HA2, which is responsible for the membrane fusion activity of HA, thereby is common designation as “fusion peptide” [4]. In its native conformation, HA monomers fold into two structurally distinct domains: a globular head and a fibrous stem (Figure 1.3). The globular head is entirely composed of HA1 residues and is formed by vestigial esterase (E’) and receptor binding (R) sub-domains. The stem region, more proximal to the viral membrane, contains residues from both HA1 and HA2 and is formed by F´and F fusion sub-domains [13]. The cleavage site between HA1 and HA2, which is usually a single arginine residue (monobasic cleavage site), is located in the middle of the stem [4,42]. Both E´and F’ sub-domains of HA1 form important contacts with the HA2 subunit of the protein (F sub-domain) [43].

The R HA1 sub-domain holds the receptor binding site (RBS) and the highly variable antigenic binding loops that surround it (HA antigenic determinants) [42]. The RBS is a deep cavity of conserved amino acid residues. Its base is formed by four conserved residues linked through hydrogen bounds: Y98, W153, H183, and Y195 (H3 numbering);

while its edges are formed by three conserved elements of secondary structure: the 130-loop (right edge; 133–138); the 220-loop (left edge; residues 220–229); and the 190-α-helix (top; residues 189–199) (H3 numbering) [13]. A second sialic acid binding site, for which the affinity of sialyllactose is four times weaker than that for the primary site, was identified between subunits of the HA trimer, specifically in an interface where two HA1 subunits and a HA2 subunit make close contact [44]. Its biological significance is still unknown [4].

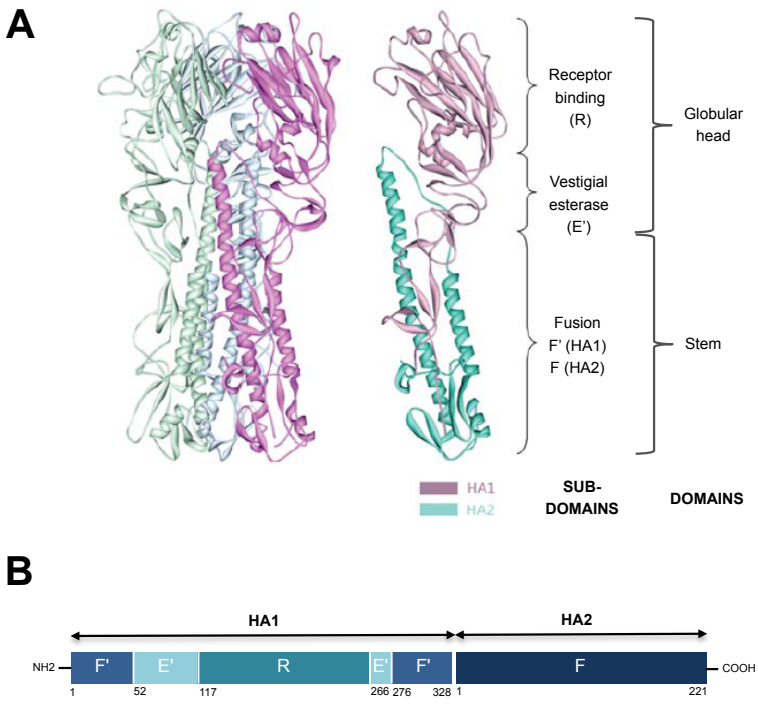

Figure 1.3 Structural features of influenza virus hemagglutinin.

(**A**) Native conformation of the hemagglutinin (HA) homotrimer (left) and of a HA monomer (right). The two HA subunits, HA1 and HA2, are coloured differently in the HA monomer, on which are also indicated the different structural domains and sub-domains of HA. Adapted from Ge *et al.* [45]. (**B**) Schematic diagram of an unfolded HA monomer from an influenza A H3 virus, evidencing the different structural sub-domains within HA1 and HA2 subunits: R - receptor binding (HA1 residues 117-265); E´ - vestigial esterase (HA1 residues 52-116, 266-275); F' - fusion (HA1 residues 1-51, 276-238); F – fusion (HA2, residues 1-221) [46].

1.1.3.2 M2 Protein

M2 protein is a single-span membrane protein specific of influenza A viruses that tetramerizes (disulfide-linked homotetramer or two dimers linked by disulfide bonds) in the viral membrane to form pH-activated proton-selective channels [47,48]. It plays its major

role during virus entry by conducting protons from the acidified endosomes into the interior of the virus, facilitating the low pH dissociation of the viral RNP complexes from M1 and its subsequent release into cytoplasm as free structures ready for nuclear import [49]. In highly pathogenic avian influenza viruses (H5 and H7), M2 is also essential during viral assembly to increase the pH of the host-cell trans-Golgi network and prevent premature low pH-induced conformational changes in HA that would inhibit virus release [5]. Recently, it was suggested that M2 protein might also play a role in recruiting the newly synthetized M1 to the cell surface during virus budding [48].

Each M2 monomer consists of three structurally distinct domains: a short and highly conserved N-terminal ectodomain (residues 1-23); a transmembrane domain (residues 24-46); and a C-terminal cytoplasmic tail with palmitate and phosphate modifications (residues 47-97) [48,50]. Figure 1.4 shows the M2 protein in its native state (homotetramer), evidencing the three structural domains of each monomer.

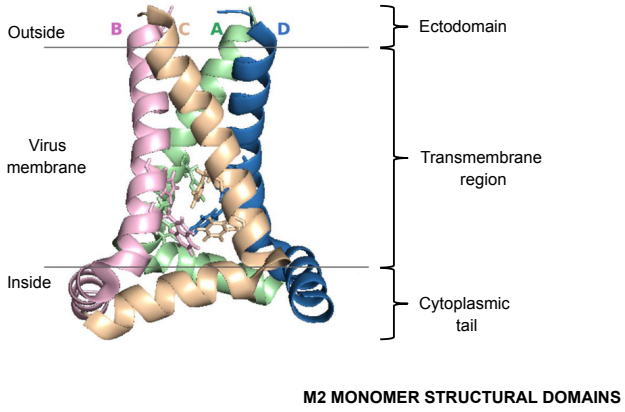

Figure 1.4 Structure of the M2 protein homotetramer.

The four monomers of the M2 protein are coloured differently and coded from A to D. At the right side are indicated the different structural domains comprising each monomer. Adapted from Martinez-Gil and Mingarro [51].

1.1.4 Evolutionary Mechanisms

Influenza viruses are characterized for evolving rapidly and unpredictably. The molecular mechanisms underlying its evolution include mutation, reassortment and, in

rare instances, recombination. Insertions and deletions in HA and NA genes have also showed to play a role in virus evolution, particularly in type B viruses [52].

Random mutations (antigenic drift)

Influenza virus RNA polymerase has no proofreading activity, resulting in a high mutation rate during replication, of approximately one error per replicated genome (error rate >$7.3x10^{-5}$ per nucleotide position per replication cycle, influenza A virus polymerase) [53,54]. The gradual accumulation of mutations in the genome segments encoding HA and NA surface proteins may result in minor amino acid changes in their antigenic sites that cause the virus to escape recognition by neutralizing antibodies and allow it to cause seasonal epidemic outbreaks. This phenomenon is called antigenic drift [10]. Antigenic drift is responsible for the need to continually review and periodically update (approximately every 2 to 5 years) influenza vaccine composition [55]. Different patterns of antigenic drift have been identified among circulating human influenza viruses. Influenza A(H3N2) viruses have shown to evolve more rapidly, with new drift variants emerging more frequently and completely replacing the older ones (single lineage); while antigenically distinct variants of influenza A(H1N1) and B viruses often co-circulate (multiple lineages), reflecting a slower emergence of new drift variants [56]. Recently emerging A(H1N1)pdm09 viruses have not yet drifted significantly, probably due to the lack of selective pressure in a predominantly naive population [57].

Reassortment events (antigenic shift)

Reassortment is the rearrangement of viral genome segments in cells infected with two (or more) different influenza viruses. It occurs in all different types of influenza viruses (A, B, and C), but has not been observed among them [10]. Reassortment events have occurred frequently among co-circulating human influenza viruses. For example, it occurred between different influenza A subtypes - H1N1 and H3N2, giving origin to the A(H1N2) variant that circulated between 2000 and 2002; and between the two influenza B lineages, resulting in the B/Yamagata (YAM) HA: B/Victoria (VIC) NA variant that predominated and became the currently circulating B/VIC-lineage virus [58]. In influenza A viruses, reassortment events may result in major antigenic changes in which an HA or NA antigenically distinct from the circulating variant is introduced into the human population. This phenomenon is called antigenic shift [10]. Antigenic shift variants have the potential to cause an influenza pandemic (i.e. epidemic of unusually large proportions [59]),

as the human population has little or no immunity against it. The last three influenza pandemics – 1957 H2N2 ('Asian flu'), 1968 H3N2 ('Hong Kong flu'), and 2009 H1N1, were caused by reassortant virus from human, avian and, in the latter case, swine origins [10]. In fact, two reassortment events were in the origin of the new 2009 H1N1 variant. All genome segments except NA and M derived from the former triple human/avian/swine reassortant virus that has been circulating in the North American pig populations since 1998 [60]. NA and M segments were introduced from the Eurasian avian-like swine H1N1 lineage. Reassortment is not the only mechanism leading to antigenic shift. Other mechanisms include: direct transmission of an avian influenza virus to humans, followed by continued adaptations (suggested for the 1918 H1N1 pandemic ('Spanish flu')) [61,62]; and, re-emergence of a previously circulating virus (1977 H1N1 virus, 'Russian flu' epidemic) [63]. Antigenic shifts are characterized for occurring suddenly and at irregular and unpredictable intervals [10].

Recombination

Recombination occurs when foreign genetic material, from another influenza virus infecting the same cell or from the cell itself, is incorporated into the viral genome. This process is though to occur through template switching during viral replication [64]. Currently, there is ample evidence that non-homologous recombination (wherein the recombining material belongs to a different genome segment or the host cell) occurs in influenza A viruses, although rarely [65-67]. The occurrence of homologous recombination (wherein the recombining material belongs to the same genome segment) continues to be proven [68].

1.1.5 Currently Circulating Subtypes and Lineages

Influenza viruses currently circulating among humans belong to two distinct influenza A subtypes - H3N2 and 2009 H1N1 pandemic, and two distinct influenza B lineages - B/VIC and B/YAM. A former influenza A H1N1 subtype circulated in the human population until recently (2011)[69] when it was replaced by the current 2009 H1N1 pandemic subtype. Both H1N1 subtypes as well as the H3N2 subtype emerged in the human population in the form of a pandemic virus. Influenza A(H1N1) viruses emerged in the 1918 pandemic, circulated until 1957 when were replaced by the new A(H2N2) pandemic virus (1957

pandemic) and then re-emerged in 1977 causing a worldwide epidemic ('Russian flu' epidemic) [70]. Influenza A(H3N2) viruses emerged in the 1968 pandemic and since then have been in continuous circulation [71]. Unexpectedly, they were not supplanted by the A(H1N1) viruses when these re-emerged in 1977, which allowed, for the first time in influenza history, two distinct influenza A subtypes to co-circulate in the human population [72]. Influenza 2009 H1N1 pandemic viruses (A(H1N1)pdm09) caused the most recent pandemic. Influenza B viruses were first identified in 1940 as a circulating homogenous group [73]. Only in the early 1980s they diverged into the two antigenically and genetically distinct lineages B/VIC and B/YAM, which were named after their first representatives, B/Victoria/02/1987 and B/Yamagata/16/1988 [74]. These two lineages have been co-circulating in the human population for more than 25 years, with the exception of the 1991-2000 decade, during which B/VIC-lineage viruses were geographically restricted to eastern Asia for reasons still unknown [75].

Figure 1.5 illustrates the periods of prevalence of all influenza A subtypes and influenza B lineages currently or until recently circulating among humans.

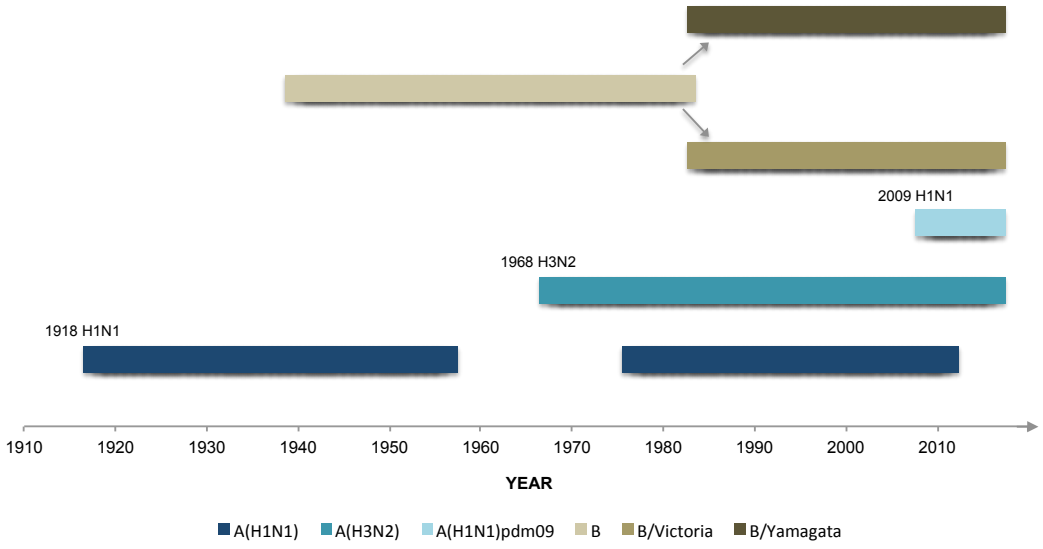

Figure 1.5 Timeline of currently or recently circulating human influenza A and B viruses.

The different influenza pandemics that originated the current influenza A viruses are indicated above the corresponding time line.

1.2 INFLUENZA VIRUS NEURAMINIDASE PROTEIN

NA is the second major surface protein of influenza A and B viruses. It is a mushroom-shaped homotetramer of non-covalently linked monomers, with the N-terminus oriented toward the interior of the virus and the C-terminus projecting as a spike away from the viral surface - type II integral membrane protein [76,77]. NA constitutes the central protein for the purpose of this thesis, as it is the target of NAI drugs.

1.2.1 Function

NA plays its major role at the final stage of viral infection. It removes α-2,3 or α-2,6-linked sialic acid moieties from host-cell receptors and newly synthesized HA and NA proteins, facilitating the release of progeny virions and promoting its spread towards neighbouring uninfected cells [78]. As NA's function opposes to that of HA (binding to host-cell receptors), the two surface proteins work in concert during virus infection to ensure an optimum balance between their receptor-binding and receptor-destroying activities [79]. This balance is essential for efficient virus replication. NA also exerts its sialidase activity on the mucus that covers the respiratory tract epithelium (rich in sialic acid), cleansing the environment and facilitating the access of virus particles to host cells, promoting virus infection [49]. It has also been suggested that NA may play a role in assisting virus entry and/or enhancing late endosome/lysosome trafficking [5]. Besides these key functions on virus infection, NA is also the secondary surface antigen of influenza viruses, eliciting the production of specific antibodies (host´s antibody-mediated immune response). Although they do not neutralize virus infectivity as the antibodies produced against HA (major surface antigen), anti-NA antibodies can ameliorate disease severity [41]. Antibody epitopes in NA are not, however, so well characterized as they are in HA, particularly in influenza B viruses. NA functions are summarized above in the Table 1.1 (section 1.1.3).

1.2.2 Structural Domains and Enzyme Active Site

Each NA monomer is comprised by a single polypeptide chain of approximately 470 amino acids, arranged into four functional domains: a short N-terminal cytoplasmic tail, followed by a hydrophobic transmembrane region and a thin stalk, ending in a globular

head domain that carries both the catalytic center (enzyme active site) and antigenic sites [80] (Figure 1.6).

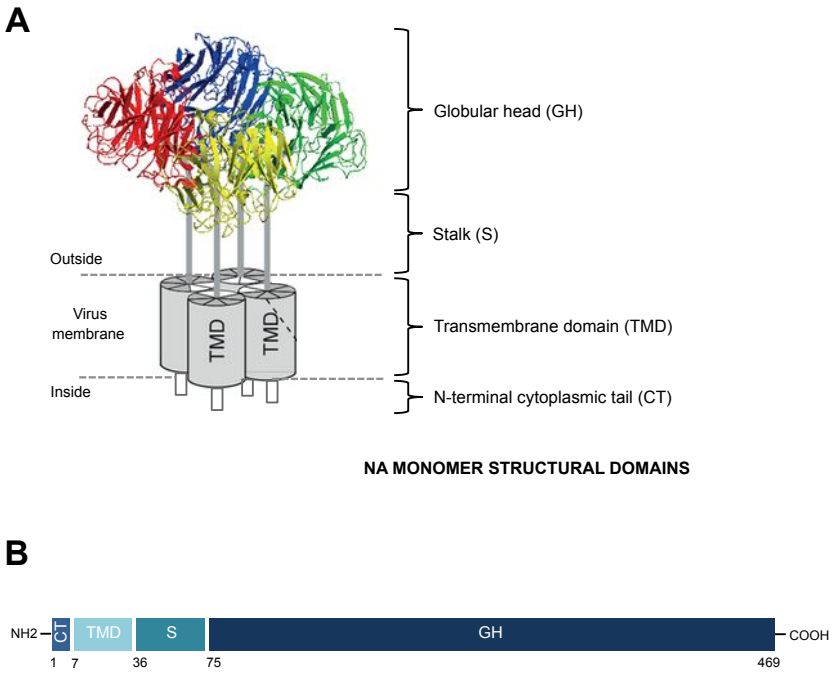

Figure 1.6 Schematic representations of the neuraminidase homotetramer (**A**) and of an unfolded neuraminidase monomer (**B**), with indication of the different structural domains.

Panel A: the four structural domains of each neuraminidase (NA) monomer are indicated on the right side. The catalytic globular head domain is additionally coloured, with each colour representing a different monomer. Adapted from da Silva *et al.* [81]. **Panel B**: NA monomer from an influenza A N2 virus. The different coloured rectangles represent the different structural domains: CT – cytoplasmic tail (residues 1-6); TMD – transmembrane domain (residues 7-35); S – stalk (residues 36-74); GH – globular head (residues 75-469) [82].

The N-terminal cytoplasmic domain is a sequence of 6 polar amino acids, highly conserved across all influenza A NA subtypes (MNPNQK) but not in influenza B NA [76]. It has an important role in the incorporation of the NA into progeny virions but it is not essential for virus replication [4]. The transmembrane domain consists of 29 hydrophobic amino acids (30 and 31 amino acids solo in N8 and N5 NA subtypes, respectively), extremely variable among subtypes and predicted to form a transmembrane helix in all of them [80,83]. This transmembrane sequence has a combined signal peptide-anchor function of directing the NA across the endoplasmic reticulum and retaining it in the viral membrane [80]. The stalk is the most variable domain, varying not only in sequence but also in length, both among and within subtypes [82]. Most NAs have stalks of approximately

50 amino acids, but deletions of up to 18 amino acids have been found in N1 and N2 NA [80]. Changes in the stalk length are suggested to regulate the distance of the enzymatic globular head to host-cell receptors [84]. The structure of the NA stalk is, however, still unknown. The enzymatically and also antigenically active globular head domain contains the largest number of conserved amino acids [85]. Approximately 390 amino acids comprise this C-terminal domain.

The enzyme active site presents as a deep pocket on the center of the globular head. It is formed by 8 functional residues that interact directly with the sialic acid molecule – R118, D151, R152, R224, E276, R292, R371, and Y406 (N2 numbering); and 10 framework residues implicated in the stabilization of the active site structure – E119, R156, W178, S179, D198, I222, E227, E277, N294, and E425 (N2 numbering) [13]. These 19 residues are highly conserved in all influenza A and B viruses and mutagenesis studies in these specific positions have mainly resulted in the loss of enzymatic activity [86]. Due to its highly conserved structure and its critical role in virus infection and spread, the active site constitutes the target of NAIs. Although located independently in the globular head of each individual NA monomer, the active site is only enzymatically active when NA adopts its mature form of homotetramer [77].

1.2.3 Group-Specific Structural Features

The currently known influenza A NA subtypes (N1 - N11) cluster into three distinct genetic groups, known as group 1, group 2 and influenza A-like group 3. Group 1 includes N1, N4, N5 and N8, while N2, N3, N6, N7 and N9 belong to group 2. The recent bat-derived N10 and N11, considered as NA-like molecules due to the lack of sialidase activity, form the influenza A-like group 3 [87]. Influenza B NA is genetically far away from all known influenza A NA subtypes, clustering in a separate and homogenous genetic group [88]. The genetically distinct NA groups have also distinctive structural features. One of the most notable is displayed by group 1 NAs, on which the 150-loop of the active site, located between residues 147 to 152 (N2 numbering), adopts an exceptional open conformation, leading to formation of an additional cavity adjacent to the active site - 150-cavity [88]. In all other NAs this loop is otherwise in a close conformation (150-cavity deficiency). The 2009 N1 pandemic is an atypical group 1 NA as it lacks the characteristic 150-cavity [89]. The influenza A-like group 3 NAs are distinguished by the unique

architecture of their active site that is present in a more open conformation unfavourable for the binding of the sialic acid molecule, which may explain the lack of sialidase activity [87]. Only minor features characterize the structure of influenza B NA. Of notice, the floor of its active site is more sterically crowded, which indicates that the residues might be tightly constrained to the observed positions in the uncomplexed enzyme [90].

1.2.4 Evolution and the Underlying Role of Positive Selective Pressure

NA has been evolving rapidly in all human influenza virus subtypes or lineages, at a fixation rate that varied from 1.79 to 3.12 $x10^{-3}$ nucleotide substitutions per site per year (subs/site/year) in former seasonal A(H1N1) viruses (herein designated as seasonal A(H1N1)) [91,92], from 2.28 to 3.27 $x10^{-3}$ subs/site/year in A(H3N2) viruses [92-95], and from 1.90 to 2.04 $x10^{-3}$ and 2.25 to 2.30 $x10^{-3}$ subs/site/year in, respectively, B/VIC and B/YAM-lineage viruses [92,96]. In 2009 A(H1N1) pandemic viruses, the fixation rate was estimated to be of $2.83x10^{-3}$ subs/site/year [92]. Recently, it was found that NA has been evolving gradually in A(H3N2) viruses and not episodically as HA (sporadic bursts of evolution) [97].

As a surface antigen, NA is under constant selection of the host's immune system (antibody-mediated immune response). Positive selection of amino acid variants that allow the virus to escape host immunity (antigenic drift; see section 1.1.4) is one of the major forces driving the evolution of NA in influenza A and, to a lesser extent, influenza B viruses [98]. Natural selection operating in influenza virus NA can also arise from interactions with other viral genes, stability of the protein structure and adaptation to new species [99]. The worldwide introduction of NAI drugs into clinical practice in 1999 created an additional source of selection – drug use, changing the environment on which NA was evolving. Only recently was, however, given a higher attention to the selective pressure (SP) forces acting on NA, particularly on the N1 NA of 2009 A(H1N1) pandemic viruses. Until 2010, virtually all studies investigating SP on influenza viruses were focused on HA due to its role of major surface antigen (primary target of immune response). The ratio between the rates of non-synonymous (dN) and synonymous (dS) substitutions (dN/dS ratio) constitutes the standard measure of SP. A dN/dS ratio significantly less than one indicates negative selection; $dN/dS \approx 1$ represents neutral

evolution; and a dN/dS ratio significantly greater than one provides evidence of positive selection [100]. Global estimates of the dN/dS ratio on NA gene have been lower than one for all human influenza virus types and subtypes or lineages (0.19 to 0.31) [91,92,94-96,98,101-104], indicating overall negative selection. Similar results have also been obtained at site-by-site specific analysis. Many NA sites were found under negative selection and only a few of them (1 to 16 sites (or 9 under the agreement of at least two methods)) [91,92,94-96,98,101,102,104-106] or even none (2009 N1 pandemic subtype [107]) were found to be experiencing positive selection. Moreover, all or most positively selected sites were functionally important, belonging to antibody recognition sites (344 and 365 in N1 [92]; 199, 328, 334, 338, 367, 370 and 401 in N2 [94,95,104]; 396 in NA B [98]; and 249 in N1pdm09 [105]), or being involved in either sialic acid/NAI drug binding (151 in N1 and N2; 373 and 374 in NA B [92]), calcium binding (379 in N1; 345 in NA B [92]), or host adaptation (46 in N1pdm09 [101]). Positive selection was also detected at seasonal and 2009 pandemic N1 NA site 275 associated with drug resistance [102,105]. Most SP studies have, however, only included a small number of representative NA coding sequences (75 to 345 sequences) and/or covered short periods of time (1 to 11 years). Also, none of the studies gave a complete picture of the SP acting on all different sites of influenza NA (overall site-specific selection map), essential to known the strength of SP acting at other sites than those positively and/or negatively selected.

Importance:

SP studies are essential for a better understanding of the evolutionary dynamics of influenza NA. Moreover, negatively selected sites may constitute potential new targets for antiviral drugs, as many of the amino acid substitutions occurring at these sites are likely to be intolerable; while positively selected sites may be useful to identify key immune epitopes responsible for protective immunity in influenza virus infections [104].

1.3 CLINICAL DISEASE AND EPIDEMIOLOGICAL FEATURES

1.3.1 Clinical Disease and its Impact

Influenza virus causes a respiratory illness in most infected people [108]. The spectrum of clinical infection is broad, varying from relatively mild or even asymptomatic virus

shedding to severe illness, pneumonia, and death [109]. Clinical symptoms are also varied and highly diverse (fever, cough, sore throat, malaise, muscle fatigue, headache, etc.), lacking specificity as are not easily distinguished from those caused by other respiratory pathogens, such as respiratory syncytial virus or human metapneumovirus [108]. The term influenza-like illness (ILI) is therefore used in the clinic to designate the disease based on exclusively its non-specific symptoms. Current World Health Organization (WHO) definition of ILI includes the presence of fever ≥ 38 C° and cough with onset within the last 10 days [110]. Upon laboratory confirmation, clinical diagnosis of influenza virus infection becomes definitive. Influenza A and B viruses cause the same spectrum of disease but the frequency of severe influenza B virus infections requiring hospitalization is about fourfold less than for influenza A virus [10]. Persons at higher risk of developing severe disease include: infants and young children; the elderly; persons with comorbidities, particularly asthma and other chronic cardiopulmonary diseases; pregnant women; morbidly obese patients; and, immunosuppressed patients [108].

Influenza virus infections cause significant morbidity and mortality worldwide. Recent WHO global estimates indicated the occurrence of approximately 1 billion cases of influenza virus infection per year, with around 3 – 5 million cases of severe illness, and 300 000 – 500 000 deaths [111]. Annual influenza epidemics have also a substantial economic impact associated to the lost or reduced productivity in the work place or at home due to incapacitating illness and the increased health-care costs from treating the disease [112]. Robust estimates of the annual economic burden of influenza epidemics in the United States amounted to $87.1 billion [113]. Both public health and economic impacts are even greater in the case of influenza pandemics. The 1918 H1N1 pandemic was the most devastating pandemic in recent history, having affected one third of the world's population (~500 million persons) and caused approximately 50 million deaths [114].

1.3.2 Transmission and Seasonality

Human influenza virus is primarily transmitted from person to person via virus-laden respiratory secretions generated when infected persons cough, sneeze and possibly talk. Transmission may also occur through hand contamination from fomites followed by self-inoculation into the nose, mouth or perhaps eyes [108]. Its easy way of transmit makes

influenza a highly contagious disease, even more in a crowded and connected world as that as we live today.

Human influenza infections exhibit a clear seasonal cycle in temperate regions [115]. They occur at a low level throughout the year and only increase markedly during the winter months – winter seasonality [116]. As the northern and southern hemispheres have winter at opposite times of the year, there are actually two different influenza seasons each year. Northern Hemisphere influenza season is generally from October to May, with peak activity in January or February, while in the Southern Hemisphere is usually from May to October, with peak activity in July or August [117]. It is still not clear why annual human influenza epidemics follow this seasonal pattern in temperate climates. In tropical and subtropical regions, human influenza epidemics often occur during the rainy season or transmit year-round without a well-defined season [115].

1.4 INFLUENZA ANTIVIRALS

1.4.1 Key Role in Influenza Management and Control

Annual vaccination is the mainstay of influenza prevention but it offers only limited protection for some patient groups, such as the frail elderly and immunocompromised individuals, and circumstances (e.g. poor match between the vaccine and circulating viruses). Also, there are some individuals that due to specific contraindications cannot be immunised (e.g. egg allergy) [118,119]. In these particular situations, antiviral therapy can be a useful complement or alternative to vaccination for preventing influenza (pre-exposure prophylaxis). Following virus infection, vaccination is no longer a viable intervention and antiviral drug use becomes the leading strategy to combat influenza, either by treatment or post-exposure prophylaxis [118].

Antivirals can be especially helpful in controlling influenza outbreaks in confined populations at increased risk of severe illness, such as the elderly in nursing homes [120]. By treating those who are already infected, antivirals reduce the probability of secondary transmissions and help preventing the spread of infection [121]. They also play a key role in the frontline defence against an influenza pandemic outbreak, particularly during the early months when a specific vaccine is unavailable, as recently evidenced during the

2009 H1N1 pandemic [122]. Hence, many countries continue to stockpile antiviral drugs as part of their preparedness plans for a next influenza pandemic [123]. People with severe influenza infections or at higher risk of complications from influenza (high-risk groups) constitute the main target population for antiviral therapy [124].

1.4.2 Approved Antivirals

Presently there are two classes of influenza antiviral drugs approved almost universally for human use: the M2 protein inhibitors (also designated as adamantanes; herein designated as M2 inhibitors) and the NAIs [125]. The M2 inhibitor drug class was the first to be approved, dating from the 1960s. It includes amantadine (Symmetrel®, Endo Pharmaceuticals Inc.; and other generic equivalents), and its analog, rimantadine (Flumadine®, Forest Pharmaceuticals Inc.; and other generic equivalents), both orally available [126]. The NAI drug class was introduced in 1999 and now includes four compounds: zanamivir (Relenza®, GlaxoSmithKline Inc.); oseltamivir phosphate (Tamiflu®, F. Hoffmann-La Roche AG; herein designated as oseltamivir); peramivir (Rapivab®, BioCryst Pharmaceuticals Inc.); and laninamivir octanoate (Inavir®, Daiichi Sankyo; herein designated as laninamivir) [125]. Oseltamivir (OS) and zanamivir (ZA) were the first NAIs to be available, in oral and inhaled formulations, respectively, both dating from 1999. Moreover, they are the only NAIs approved worldwide [49]. Peramivir (PER) is only approved in limited markets that include Japan (2010; as Rapiacta®), the Republic of Korea (2010; as Peramiflu®) and, very recently, China (2013) and the United States of America (USA) (2014). It constitutes the only intravenous option for treating influenza. Laninamivir (LAN) is approved for human use exclusively in Japan (2010) [10,127,128], being available as an inhaled NAI with long-acting properties.

A third class of influenza antiviral drugs - HA fusion inhibitors, is approved in Russia since 1993 and China since 2006. It includes a single compound – arbidol hydrochloride (commonly designated as arbidol), orally administrated [129]. Due to its limited approval, this antiviral drug class will not be further discussed in this review.

In Portugal, amantadine is approved as anti-influenza agent since 1973 (as Parkadina®), while OS and ZA were introduced in, respectively, 2002 and 1999 [130].

OS is the most widely used influenza antiviral drug and is now on the WHO List of Essential Medicines, a list that includes the most important medications needed in a basic health system [131]. It is also the main antiviral drug that has been incorporated into national, international, and private stockpiles for pandemic preparedness [123].

Intravenous formulations of OS and ZA are now in clinical development [49]. These will be especially important for severely ill patients who cannot take oral or inhaled medication, such as those mechanically ventilated [123]. In fact, intravenous ZA is already available via clinical trial or compassionate use request [132] and has been used in critically ill immunosuppressed adults and children [133]. Other investigational anti-influenza agents in late-phase clinical trials target other viral proteins or host factors and include favipiravir (virus RNA polymerase), and nitazoxanide and fludase (host) [125]. Recently, favipiravir (Avigan®, Toyama Chemical Co.) was approved in Japan for influenza pandemic preparedness [134].

1.4.2.1 M2 Protein Inhibitors

Amantadine (AMA) and rimantadine (RIM) were not specifically designed as anti-influenza drugs. They were developed serendipitously and only later was discovered its activity against influenza. In fact, only after almost 25 years of their development it was found that they target the M2 protein of influenza A viruses [135]. Importantly, they are not effective against the analogous BM2 channel of influenza B viruses, which makes them a specific anti-influenza A agent [49]. Besides influenza, AMA is also active against human Parkinson's disease and since early (1973) is also approved for its treatment [136].

AMA and RIM are synthetic tricyclic amines. Chemically, AMA is 1-amino adamantane hydrochloride and RIM is its α-methyl derivative (α-methyl-1-adamantane methylamine hydrochloride) [137]. As structural analogues, they share mechanisms of action and pharmacological profiles.

1.4.2.1.1 Mechanism of action

AMA and RIM block the acid-activated proton channel formed by the M2 protein of influenza A virus (detailed in section 1.1.3.2). By blocking this channel, they stop the flow

of H+ ions from the acidified endosome into the interior of the virion and thereby inhibit the dissociation of the viral RNP complexes from the M1 protein and its subsequent release into the cytoplasm - inhibition of viral uncoating [10] (Figure 1.7). In highly pathogenic avian influenza viruses (H5 and H7), they further prevent the pH increase in the host-cell trans-Golgi network, necessary for HA maintain its conformation during the transport to the virus membrane [49].

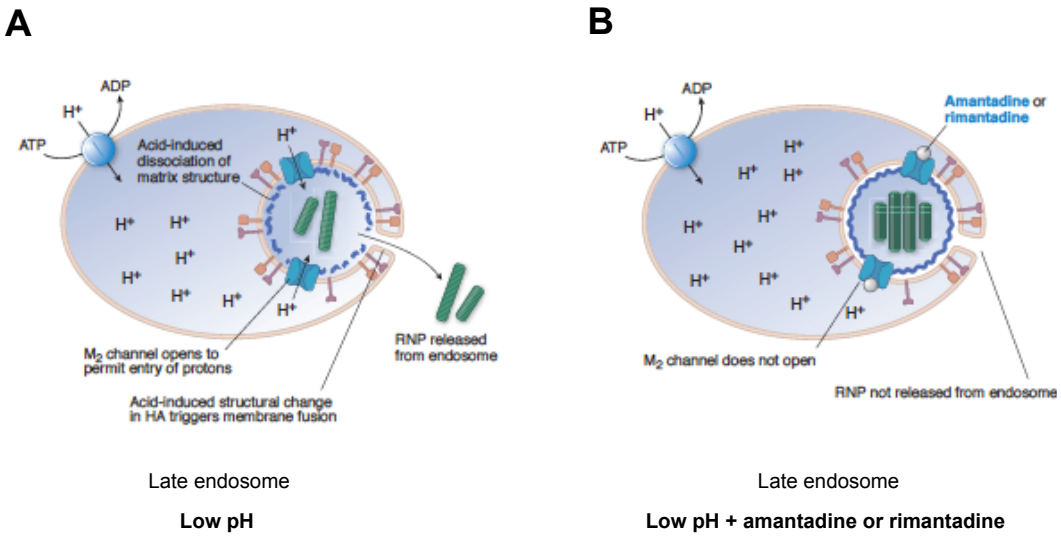

Figure 1.7 Schematic representation of the process of (**A**) influenza A virus uncoating and (**B**) drug action of amantadine and rimantadine.

Panel A: The acidic environment of the endosome, as a result of the flow of H+ protons from the cytosol into the endosome by a H+-ATPase, triggers conformational changes in hemagglutinin (HA) that expose the fusion peptide, promoting the fusion of viral and endosome membranes. This fusion event is not sufficient to cause viral uncoating. In addition, H+ protons from the late endosome must enter the virion through the M2 proton channel, which opens in response to acidification (pH-induced channel). The entry of H+ protons into the virion causes dissociation of the matrix protein (M1) from the influenza A virus ribonucleoprotein (RNP) complexes, releasing them into the cytoplasm. The dissociation of M1 is required for RNP complexes to be transported to the cell nucleus where they are transcribed and replicated. **Panel B**: Amantadine and rimantadine block the M2 proton channel function and thereby inhibit acidification of the interior of the virion, M1 dissociation and virus uncoating. Adapted from Coen and Richman [138].

The mechanism by which AMA and RIM block the M2 proton channel was only recently elucidated by the study of Pielak *et al.* [139]. After years of controversy, Pielak and colleagues reported a nuclear magnetic resonance (NMR) structure of RIM bound inside the pore of a drug-sensitive chimeric A/M2 – BM2 protein. This novel NMR structure supported the results of previous mutational [140] and X-ray crystallographic structural [141]

studies, confirming that the drug binds inside the pore and physically occludes the channel. Specifically, it binds to a hydrophobic pocket near the N-terminal end of the channel, formed by the methyl groups of residues V27 and A30 from the four subunits, with the amino group of the drug appearing to be in polar contact with the backbone oxygen of A30 residues [139]. The binding occurs when the channel is in its closed state [48].

1.4.2.1.2 Efficacy and use

M2 inhibitors can prevent approximately one-half of influenza A virus infections and 70 to 90% of illnesses when used as prophylaxis [142]. Its treatment showed also to be beneficial, shortening the duration of fever by 1-day on average when administered within 48h of symptoms onset [143]. Also, it revealed to be associated with more rapid symptom resolution, functional recovery, and, in some studies, resolution of small airways functional abnormalities [144].

M2 inhibitors have now been available for about 50 years. Its use has been very little in most countries around the world [145], but in some of them, such as China and Russia, they have been available as over-the-counter drugs and have been even included in various cold medicines that do not need a prescription [146]. Concerns about the possible development of adverse effects, particularly in the elderly, have limited the use of these drugs [147]. Both AMA and RIM use is associated with several central nervous system (CNS) side effects, with other major side effects including gastrointestinal disorders and anti-muscarinic effects [142,148]. The lack of demonstrated prevention of complications has also limited their use, although in a lesser extent [147], with the rapid selection of drug-resistant variants that could spread efficiently in the presence or absence of drug constituting the major threat to their use. In fact, since 2010 M2 inhibitors are no longer recommended by WHO for prophylaxis and treatment of influenza virus infections due to the overall resistance observed [124] (see section 1.5.1.3 for detail).

1.4.2.2 Neuraminidase Inhibitors

NAIs were design to mimic the natural substrate of influenza A and B virus NA enzyme - sialic acid (N-acetylneuraminic acid, Neu5Ac), and to compete for the binding at the

active site. Based on the structure of a transition-state analogue of sialic acid, DANA (chemically 2-deoxy-2,3-dehydro-N-acetylneuraminic acid), the NAIs possess higher binding affinity than the Neu5Ac, preventing the cleavage of the natural substrate [149].

ZA is a 4-deoxy-4-guanidino analog of DANA, resulting from the single substitution of the hydroxyl at the 4' position on the sugar ring with a guanidino group [150]. OS is otherwise more distinct, possessing a cyclohexene ring instead of the sugar ring and two further substitutions: an amino group replacing the hydroxyl at the 4' position and a large hydrophobic pentyl ether group replacing the glycerol side chain at the 6' position. OS is administered in the form of a prodrug (oseltamivir phosphate, OSP) that is converted by hepatic esterases into its active form, oseltamivir carboxylate (OSC) [150]. The high bioavailability of OSP allowed it to be the first NAI orally administrated [151]. PER and LAN were both design most recently. PER is still directly based on DANA, having a cyclopentane ring instead of the sugar ring and both the 4-guanidino group of ZA and the 6-hydrophobic pentyl ether group of OS [150]. LAN is based on ZA, resulting from the single substitution of a hydroxyl group on the glycerol side chain with an acyl group [152]. Similarly to OS, LAN is administrated as a prodrug (laninamivir octanoate, LANO) that is converted in the lungs into LAN [148]. A single administration of LANO results in a long retention of the drug in the lungs, conferring a long-lasting anti-NA activity (long-acting NAI drug) [150]. The chemical structure of all NAIs is shown below in Figure 1.8.

1.4.2.2.1 Mechanism of action

NAIs act by binding to the active site of the virus NA protein and blocking its sialidase activity (detailed in section 1.2.1). As a result, progeny virions fail to be released from sialic acid receptors and aggregate on the surface of the infected cell, hampering the spread of infection to neighbouring uninfected cells - inhibition of viral release and spread [153] (Figure 1.8). The influenza virions entering the respiratory tract fail also to be released from sialic acid receptors and remain trapped in the mucus, making it difficult to access the target cells and initiate virus infection [154].

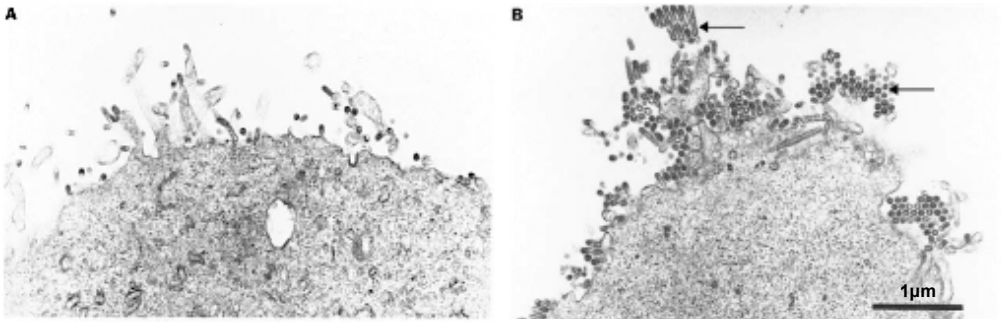

Figure 1.8 Electron micrographs of Madin-Darby canine kidney cells infected with influenza A virus. (**A**) Normal assembly and budding of virus in the absence of neuraminidase inhibitor (NAI); (**B**) Lateral aggregation and formation of large bundles by virus (indicated by arrows) in the presence of NAI. Source: Gubareva *et al.* [155].

Due to differences in their chemical structure, the 4 NAI drugs bind slightly differently to the enzyme active site, as evidenced in Figure 1.9, in which are highlighted the main chemical groups involved in drug binding.

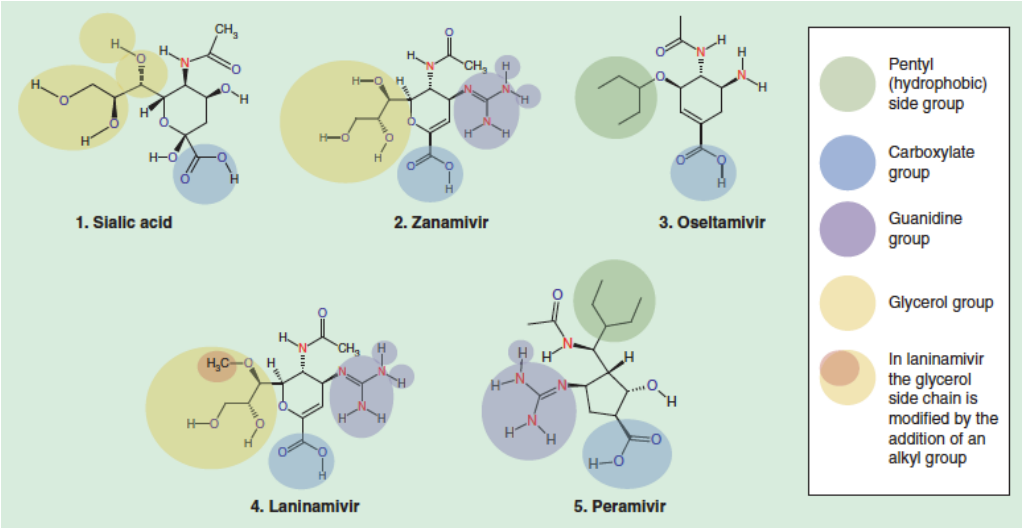

Figure 1.9 Chemical structures of sialic acid (**1**) and the neuraminidase inhibitors zanamivir (**2**), oseltamivir carboxylate (**3**), laninamivir octanoate (**4**), and peramivir (**5**).
The chemical groups responsible for the major interactions with the enzyme active site are highlighted. Source: Farrukee *et al.* [152].

Similarly to sialic acid, ZA has a carboxylate group that interacts with three positively charged residues (R119, R292 and R371) and a glycerol group that interacts with residue E276 at the active site [152]. Its 4-guanidino group creates an extra binding interaction with a pocket formed by acidic residues E119, D151, and E227 at the base of the active site,

increasing the affinity of binding in about 1000-fold [148]. The large 6-pentyl ether group of OS binds to a hydrophobic pocket that is exposed by the reorientation of residue E276 upon interaction with the inhibitor, with a similar affinity of binding of ZA. As PER possesses both 4-guanidino group of ZA and 6-pentyl ether group of OS, it binds to both pockets, being a potent inhibitor of NA [49]. LAN has a similar mode of binding than ZA [156]. The slightly different ways in which the 4 NAIs bind to the active site may result in different antiviral activities.

1.4.2.2.2 Efficacy and use

ZA and OS can prevent around 70 to 90% of illnesses when used as either post-exposure prophylaxis for close contacts, such as household members, or as seasonal prophylaxis in the community for otherwise healthy adults and children [49]. However, recent data from 2009 A(H1N1) pandemic, wherein OS was mainly used, evidenced the occurrence of asymptomatic infections and the development of drug resistance during or after post-exposure prophylaxis. Early NAI treatment is therefore now preferred over post-exposure prophylaxis [49]. NAI treatment is increasingly more beneficial as early as it is instituted, particularly within the 48h of the onset of symptoms. In healthy, ambulatory adults and children, ZA and OS treatment significantly reduce the duration of illness (1–3 days ZA; 0.5–4.1 days OS) when started within 48h after symptom onset. Drug treatment has also been associated with a significant reduction in the use of antibiotic therapy for lower respiratory tract complications and/or sinusitis (ZA) or otitis media (OS) [148]. Additionally, it apparently reduces the amount of virus shed [10]. Recent data from PER and LAN clinical studies showed treatment efficacy results comparable to those found for the former NAIs [157-160]. OS is apparently less effective for the treatment of influenza B virus infections compared with influenza A [152]. The same cannot yet be concluded for the remaining NAIs (ZA, PER, and LAN), as the number of clinical studies comparing influenza A versus influenza B data is still very limited. Nevertheless, the significantly lower susceptibility that influenza B viruses have been exhibiting *in vitro* to all NAIs supports a similar difference on clinical effectiveness [152]. Clinical data regarding NAI therapy on high-risk patients is also very limited, but observational studies have shown improved clinical outcome, including reduced mortality [49,126].

Over the years there has been a gradual increase in the use of NAIs, with its highest peak during the 2009 H1N1 pandemic. Their use has also varied widely across countries, reflecting different national public health policies [161]. Countries such as Japan and the USA use the greatest volumes and regularly treat influenza-infected patients presenting at either general practitioners or hospital clinics; while most worldwide countries, including Portugal, use only little volumes of NAIs to essentially treat severely ill hospitalised patients [125]. Aside from the treatment of seasonal influenza, many countries have stockpiled large volumes of NAIs, mainly OS, as part of their pandemic preparedness plans. All NAIs are generally well tolerated, with only minor side effects reported [126,148]. OS remains the most prescribed influenza antiviral drug, mainly by its ease mode of administration (oral formulation), with its sales exceeding those of ZA by at least 10-fold. In Japan the use of LAN has recently surpassed that of OS [145].

1.5 ANTIVIRAL RESISTANCE

As with any antimicrobial agent, the development of resistance against M2 inhibitors and NAIs can indicate that their therapeutic benefits are reduced or even abrogated, rendering them useless for treating or preventing influenza [145]. Considering that the repertoire of both M2 inhibitors and NAIs is very limited, the emergence of drug-resistant influenza variants can pose a serious threat to global public health.

1.5.1 Resistance to M2 Protein Inhibitors

1.5.1.1 Mechanisms of Resistance

The molecular basis of influenza A virus resistance to M2 inhibitors has been well characterised and is associated with amino acid substitutions at 5 key residues within the transmembrane (TM) domain of the M2 protein – residues 26, 27, 30, 31, and 34 [146]. All 5 residues are located in the N-terminal portion of the TM region (Figure 1.10), but while residues 27, 30 and 34 are pore-lining, residues 26 and 31 are rather in the helix–helix packing interface [50,162]. So far, 7 amino acid substitutions have been reported to confer M2 inhibitor resistance, including L26F, V27A, A30T, A30V, S31N, S31D and G34E -

established molecular markers of resistance [163,164]. Most drug-resistant variants contain one of these amino acid changes, but variants with dual mutations have also been described (e.g. V27A/S31N). Moreover, it is observed a complete cross-resistance between AMA and RIM [146].

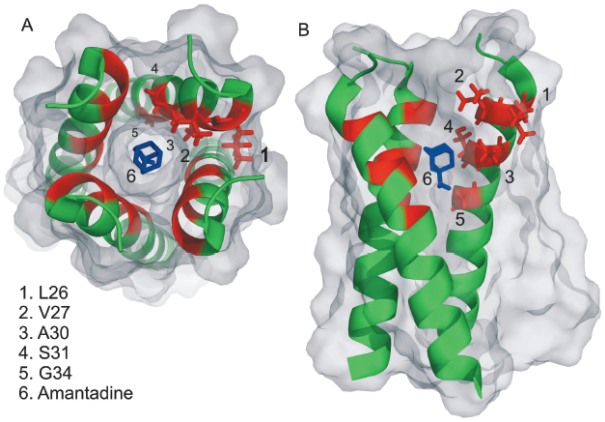

Figure 1.10 Mapping of the amino acid residues associated with M2 inhibitor resistance onto the transmembrane domain of influenza A virus M2 protein.

Top view (**A**) and side view (**B**) of the homotetrameric M2 proton channel transmembrane domain (PDB ID: 2KQT). Both protein surface (grey) and helices (green) are displayed. Residues 26, 27, 30, 31 and 34 (numbered 1-5) associated with drug resistance are displayed in red, with the corresponding side chains being displayed as sticks on a single helix only. Amantadine (number 6) is shown in blue at the proposed binding site. One of the four monomeric transmembrane domains was left out from B. The image was generated using PYMOL. Source: van der Vries [165].

Resistance-conferring mutations have been proposed to either reduce the binding affinity of the drug (V27A, A30T/V, and G34E; pore-lining residues) or cause an expansion of the M2 channel and disrupt the drug-binding pocket (L26F and S31N; helix–helix interface residues) [139]. More important than lining the interior of the pore, residues V27 and A30 are directly involved in the formation of the hydrophobic pocket to which the drug binds (detailed in section 1.4.2.1.1). Amino acid substitutions at these residues are likely to change the physical or chemical properties of the pocket and thereby affect drug affinity [139]. In fact, the V27A mutation showed to significantly reduce the hydrophobic surface of the inhibited pocket [166]. The substitution of the pore-lining residue G34 with the bulkier glutamic acid (E) may probably result in steric collision with the amino and methyl groups of the drug [139]; while bulky side chain substitutions at the helix–helix interface (L26F, S31N) may probably prevent the two adjacent TM helices from being close enough

to form the binding structure [139]. The significantly weaker TM helix-helix packing recently observed in S31N drug-resistant mutants supports this latter [167].

1.5.1.2 Characteristics of Drug-Resistant Variants

M2 inhibitor-resistant variants emerge rapidly after exposure to the drug, specifically within 2 to 5 days after initiating drug treatment. They are detectable in approximately 30% of treated immunocompetent children and adults, but in immunocompromised patients this frequency can exceed 50% due to prolonged virus replication [168,169]. A more detailed study detected resistant variants in up to 80% of AMA-treated children using molecular cloning techniques [170].

Drug-resistant variants are genetically stable, do not show impaired growth characteristics *in vitro*, and are not reduced in infectivity or virulence in animal models. Also, they cause typical influenza illness in humans and are transmissible from person-to-person under conditions of close contact [168,171].

1.5.1.3 Resistance Among Circulating Human Influenza Viruses

Until 1995, the global frequency of M2 inhibitor resistance among human influenza A(H3N2) viruses was extremely low (0.8%; virtually S31N variant), and no resistance was observed in H1N1 subtype [172,173].

Between 1995 and 2002, drug-resistant A(H3N2) viruses continued to be detected at negligible frequencies in most regions around the world, but increased slightly in frequency in China and other Asian countries (<10%) [174]. This preceded the high increase observed in 2003 and 2004, wherein drug resistance reached alarming levels in China (57.5% and 73.8,% respectively) and Hong Kong (18.3% and 69.6%, respectively). Virtually all drug-resistant viruses contained a single S31N substitution in the M2 protein [146]. During the following two years, related drug-resistant S31N viruses spread to most regions of the world, including Southeast Asia/Australasia, Japan and North America, where the frequency of resistance reached 42.1%, 65.3% and 92.3%, respectively. By 2006, virtually all A(H3N2) viruses circulating worldwide were resistant to M2 inhibitors

[174]. An increased over-the-counter use of M2 inhibitors in some regions of Southeast Asia, including China, possibly in association with the outbreaks of severe acute respiratory syndrome (SARS) and highly pathogenic A(H5N1) avian influenza virus in 2003, may have played a role in the initial local increase in resistance [175]. But, it cannot explain the rapid and global spread of drug-resistant S31N viruses that may have instead resulted from genetic hitchhiking to advantageous mutations in other segments (such as HA S193F and D225N mutations near the RBS), following a reassortment event [175]. This reassortment event, which most likely occurred in early 2005 in Hong Kong, involved a 4+4 segment exchange between drug-resistant viruses bearing the M2 S31N mutation and sensitive viruses with an antigenically A/Wisconsin/67/2005-like HA, resulting in a new lineage of M2 inhibitor-resistant A(H3N2) viruses, designated as N-lineage [175,176]. All globally circulating drug-resistant S31N viruses were found to belong to this lineage [176].

At the same time that drug-resistant S31N A(H3N2) viruses began to spread globally (2005-2006), the same mutation started to be detected in seasonal A(H1N1) viruses, at a global frequency of 15.6%. High levels of drug resistance were, however, found in several Southeast Asian countries, particularly China (77.1%), and also in Canada (33.3%) and Europe (44.8%) [177]. The most probable explanation is that, possibly in another example of mutation hitchhiking, the S31N substitution become associated with a group of viruses antigenically classified as A/Hong Kong/2652/2006-like (Hong Kong-like lineage; HA genetic clades 2A and 2C) [145,174]. During 2006, A/Hong Kong/2652/2006-like viruses began circulating more widely, thereby increasing the frequency of drug-resistant viruses in many countries around the world, reaching 100% in several of them, such as the Philippines and South Korea [174,178]. However, in 2007 a new antigenic group of seasonal A(H1N1) virus sensitive to M2 inhibitors - A/Brisbane/59/2007-like lineage (HA genetic clade 2B), emerged and began to replace the drug-resistant A/Hong Kong/2652/ 2006-like viruses [145]. By the end of 2008, the overall frequency of drug resistance had already dropped to negligible levels, as A/Hong Kong/2652/2006-like viruses became extinct [174].

Recently emerging 2009 pandemic A(H1N1) viruses were naturally resistant to M2 inhibitors. They contained the M2 gene from the Eurasian avian-like swine A(H1N1) lineage viruses that carry the S31N substitution since 1989 [145,179]. By 2011, naturally resistant A(H1N1)pdm09 viruses had completely replaced drug-sensitive seasonal A(H1N1) viruses [69], resulting in an overall M2 inhibitor resistance among circulating

human influenza A viruses. This situation has remained unchanged to the present day [174], even after more than 5 years in the absence of drug-selective pressure (M2 inhibitor use not recommended by WHO since 2010).

M2 inhibitor resistance has also been detected in potentially pandemic A(H5N1) and A(H7N9) avian viruses. But, while in A(H5N1) subtype the frequency of resistance has been varying widely according to the genetic lineage, all A(H7N9) viruses causing human infection to date have shown to be resistant to this antiviral drug class (S31N) [174].

1.5.2 Resistance to Neuraminidase Inhibitors

1.5.2.1 Mechanisms of Resistance

Resistance to NAIs can emerge as a result of amino acid substitutions in either the NA or HA gene [168]. Amino acid substitutions in NA (drug target) can decrease the binding affinity of the inhibitor, while those in HA affect virus susceptibility by disrupting the delicate HA-NA functional balance required during virus entry and release from cells for efficient replication. Specifically, they decrease the receptor binding affinity of HA, making the release of progeny virions less dependent on NA activity [168]. Since their effect is independent on how NA is inhibited, HA amino acid substitutions confer cross-resistance to all NAI drugs [180]. The molecular basis for NAI resistance is, however, not yet fully characterised.

1.5.2.1.1 Neuraminidase amino acid substitutions

NA H274Y substitution (N2 numbering) in N1 NA influenza viruses (seasonal A(H1N1), A(H1N1)pdm09 and A(H5N1)) is presently the only amino acid change known to cause clinical resistance to NAI drugs, specifically to OS – single molecular marker of resistance [181]. Several other NA amino acid substitutions, most of them influenza type- or subtype-specific and some also drug-specific, are associated with reduced NAI susceptibility *in vitro*, but its clinical impact is less clear or even completely unknown [182]. This is the case of NA H274Y substitution regarding PER susceptibility and/or in influenza type B NA.

New amino acid changes are continually being detected and added to this group. Based on the fold-change increase that these amino acid changes induced in IC_{50} (concentration of drug required to inhibit a standardised amount of NA activity by 50%; phenotypic assay), compared to sensitive wild-type viruses, their effect on NAI susceptibility is now classified into three categories: normal inhibition (NI) - fold-change increase <10 (influenza A) or <5 (influenza B); reduced inhibition (RI) - fold-change increase 10-100 (influenza A) or 5-50 (influenza B); highly reduced inhibition (HRI) - fold-change increase >100 (influenza A) or >50 (influenza B) [183]. These new categories were defined by the WHO Expert Working Group on Surveillance of Influenza Antiviral Susceptibility (AVWG) to ensure consistent reporting of NAI susceptibility data.

Table 1.2 summarizes the NA amino acid substitutions known to confer resistance or reduced susceptibility to NAIs *in vitro* in human influenza A and B viruses. It not only includes the amino acid substitutions known to occur clinically, either naturally or associated with drug use, but also those identified through surveillance activities or reverse genetic (RG) studies or selected in cell culture or *in vivo* experiments. Amino acid substitutions causing NI are also indicated if the reduction in susceptibility is equal or greater than two-fold and confirmed by RG. For consistency, amino acid substitutions are indicated using standard N2 numbering in both the table and throughout this review, unless otherwise specified.

Most amino acid substitutions affecting influenza A and B virus susceptibility to NAIs are, as expected, located in or close to the NA active site (drug-binding pocket). For some of the substitutions located distantly from this site, such as N70S, D429G, and M449V substitutions, it is difficult to understand how they may have an impact on the active site and affect the binding affinity of the drug. Structural NA differences explain the influenza virus type- or subtype-specific character of most amino acid changes and the different levels of reduction caused by a non-specific mutation on different NA backgrounds. Most virus-specific substitutions belong to type B influenza, with many of them further exhibiting lineage specificity and some causing different levels of reduction on B/VIC and B/YAM-lineage backgrounds [184]. Drug specificity is explained by structural differences among the 4 NAIs that result in different binding interactions with the NA active site, as detailed above in section 1.4.2.2.1. Some cross-reduced susceptibility is observed between OS and PER (e.g. E119V, H274Y, and N294S) and between the later and ZA

and/or LAN (e.g. E119G, Q136K/R, and Q140R) on N1 and/or influenza B NA backgrounds. The effect of most substitutions on LAN susceptibility is still missing. But, it is expected to be similar to that on ZA susceptibility based on the high structural similarity of both drugs and on the similar phenotypic profile observed for most substitutions with a known effect. NA E119V mutation in either N1pdm09 or influenza B NA and P141S mutation in influenza B NA are the most worrying substitutions as they confer reduced susceptibility to all NAI drugs available.

Table 1.2 Neuraminidase amino acid substitutions associated with neuraminidase inhibitor resistance or reduced susceptibility *in vitro* in human influenza A and B viruses.

AMINO ACID SUBSTITUTIONS CONFERRING REDUCED SUSCEPTIBILITY *IN VITRO*

Position in NA [a]			Amino acid substitution (N2 numbering)	Influenza (sub)type	Susceptibility assessed by NA inhibition assays [b] (IC_{50} fold-change increase relative to sensitive wild-type viruses)				Source of viruses/ selection with
N2	N1	B			Oseltamivir	Zanamivir	Peramivir	Laninamivir	
41	43	40	E41G	A(H3N2)	RI (12)	NI (1)	Unk	Unk	Surv
68	70	67	N68S	A(H1N1)	NI (3)	RI (31-46)	Unk	Unk	Clin/No
107	107	105	E107K [c]	B	NI/RI (4-10)	NI/RI (1-42)	RI/HRI (6-681)	NI/RI (1-12)	Clin/No; RG
116	116	114	I116R [c]	B	NI (2)	NI (2)	RI (6)	NI (3)	Surv
119	119	117	E119V	A(H1N1)	RI/HRI (15/1727)	HRI (136/2144)	HRI (5050)	Unk	RG
				A(H1N1)pmd09	RI (60)	HRI (571)	RI (25)	Unk	RG
				A(H3N2)	RI/HRI (18-2075)	NI (1-7)	NI (1-3)	NI (3-4)	Clin/OS; Surv; RG; In vitro
				B	HRI (300)	NI (2)	HRI (531)	Unk	RG
			E119A	A(H1N1)pmd09	RI (17)	RI (90)	RI (12)	RI (82)	RG
				B	HRI (918-3171)	HRI (271-12538)	HRI (5491-13780)	HRI (421-2163)	Surv; RG
			E119D	A(H1N1)pmd09	RI (25)	HRI (827)	HRI (286)	HRI (702)	Clin/ZA; RG
				A(H3N2)	NI (2)	RI (32)	NI (2)	Unk	RG
				B	HRI (>300)	HRI (>560)	HRI (>1598)	Unk	RG
			E119G	A(H1N1)pdm09	NI (3-7)	HRI (832-1306)	RI (51)	HRI (327)	Clin/ZA; RG
				B	RI/HRI (31-2273)	RI/HRI (33-4167)	HRI (5491-8089)	HRI (421-2163)	RG; In vitro/ZA
			E119I [c]	A(H3N2)	HRI (208)	RI (17)	NI (3)	Unk	Clin/OS; In vitro/No
136	136	134	Q136K [d]	A(H1N1)	NI (1)	RI/HRI (36-327)	RI (75-80)	Unk	Clin/No; Surv
				A(H1N1)pmd09	NI (1)	HRI (86-749)	HRI (143)	RI (42-45)	Surv; RG; In vitro/No
				A(H3N2)	NI (1-7)	RI/HRI (30-132)	Unk	Unk	Surv
			Q136R [c]	A(H1N1)pmd09	NI (1)	HRI (200)	HRI (234)	RI (33)	Surv
			H136Y [f]	B	NI (3)	NI (1)	RI (13)	NI (1)	Surv
140	140	138	Q140K	B	NI (1)	NI (1-2)	RI (17)	NI (1)	RG
			Q140R [c]	B	NI (1)	NI/RI (1-15)	RI/HRI (16-91)	NI/RI (1-7)	Surv; In vitro/No; RG
141	141	139	P141S [e]	B	RI/HRI (10-68)	RI/HRI (25-160)	NI/RI/HRI (1-322)	NI/RI/HRI (3-142)	Surv; In vitro/No; RG
142	142	140	G142R [c]	B	RI/HRI (9-184)	NI/RI/HRI (1-1037)	NI/RI/HRI (1-321)	NI/RI/HRI (2-1197)	Surv; In vitro/No; RG
144	144	142	Y144H	B	NI (2)	NI (1)	RI (6)	NI (1)	Surv
148	148	146	T148I	A(H3N2)	NI (6)	NI (1)	NI (1)	NI (3)	RG
151	151	149	D151E [g]	A(H1N1)pmd09	NI (3)	RI (19)	RI (14)	NI (5)	Surv
				A(H3N2)	RI (11)	NI (2)	Unk	Unk	RG
			D151A	A(H3N2)	NI (2)	RI (29)	Unk	Unk	Surv
			D151G	A(H3N2)	NI (1)	HRI (>1500)	Unk	Unk	RG
			D151V [f]	A(H3N2)	NI (8)	HRI (164)	Unk	Unk	Surv
			D151N [f]	B	NI (1)	NI (1)	RI (8)	NI (1)	Surv
152	152	150	R152K	B	HRI (100-252)	NI/RI/HRI (5-1000)	HRI (214-400)	Unk	Clin/No; Clin/ZA; Surv; RG
153	153	151	N153S	B	NI (1)	RI (7)	Unk	Unk	Surv
155	155	153	Y155H	A(H1N1)	RI/HRI (10-123)	RI/HRI (45-555)	RI (28-37)	Unk	Surv; RG; In vitro/No
198	199	197	D198E	A(H1N1)pdm09	RI (16)	NI (7)	Unk	Unk	Surv
				B	RI (8-26)	NI/RI (2-7)	RI (6-18)	NI (2)	Clin/No; Surv; RG
			D198G	A(H1N1)pdm09	RI (17)	NI (6)	NI (2)	NI (2)	RG
			D198N	B	NI/RI (4-10)	NI/RI (2-10)	NI/RI (2-5)	NI (2-3)	Clin/No; Clin/OS; Surv; RG
			D198Y	B	RI/HRI (15/57)	RI (14)	HRI (168)	Unk	Surv; RG
201	202	200	A201T [f]	B	RI (5-8)	RI (5-7)	Unk	Unk	Surv
222	223	221	I222V	A(H1N1)	NI (3)	NI (2)	NI (1)	Unk	RG
				A(H1N1)pdm09	NI (6)	NI (2)	NI (2-3)	NI (1)	RG
				A(H3N2)	NI (2/4)	Unk	Unk	Unk	RG
				B	NI/RI (4-5)	NI (2-3)	RI (7-9)	NI (2-4)	Surv; RG
			I222M	A(H1N1)	NI (8)	NI (1)	NI (1)	Unk	RG
			I222K	A(H1N1)pdm09	RI (12-39)	NI (5-6)	NI (1-4)	Unk	Surv; RG
			I222R	A(H1N1)pdm09	RI (28-45)	RI (10-12)	RI (10)	Unk	Clin/No; Clin/OS/ZA; RG
			I222L	A(H3N2)	NI/RI (9-18)	NI (2-5)	Unk	Unk	RG
				B	HRI (65-190)	NI/RI (4-13)	RI (12-49)	NI/RI (1-5)	Clin/OS; RG
			I222T	A(H1N1)pdm09	NI (6)	NI (2)	Unk	Unk	RG
				A(H3N2)	RI (16)	Unk	Unk	Unk	RG; In vivo/OS
				B	RI (5-14)	NI/RI (2-7)	RI (6-43)	NI (3-4)	Clin/No; Surv; RG
			I222N	B	HRI (92-141)	RI (5)	HRI (82-223)	NI (3-4)	RG

(Table 1.2 cont.)

	Position in NA [a]			Amino acid substitution (N2 numbering)		Influenza (sub)type	Susceptibility assessed by NA inhibition assays [b] (IC_{50} fold-change increase relative to sensitive wild-type viruses)				Source of viruses/ selection with
	N2	N1	B				Oseltamivir	Zanamivir	Peramivir	Laninamivir	
AMINO ACID SUBSTITUTIONS CONFERRING REDUCED SUSCEPTIBILITY IN VITRO	224	225	223	R224K		A(H3N2)	HRI (>4000)	RI (>50)	Unk	Unk	RG
	226	227	225	Q226H		A(H3N2)	RI (14)	NI (1)	Unk	Unk	Surv
	245-248	246-249	244-247	Del 245-248		A(H3N2)	HRI (157-222)	NI/RI (3-21)	NI (1)	NI (1)	Clin/OS; Surv; RG
	246	247	245	S246G		A(H1N1)pdm09	RI (15)	NI (1)	NI (1)	NI (1)	Clin/Surv
				S246N		A(H1N1)pdm09	NI (3-8)	NI (2-5)	NI (1-2)	Unk	Surv; RG
				A246T		B	RI (24)	RI (39)	RI (5)	NI (3)	Surv
	265	266	264	E265V		B	NI (2)	NI (<1)	RI (7-8)	NI (1)	Surv
	274	275	273	**H274Y**		**A(H1N1)**	**HRI (321-2597)**	NI (1-2)	HRI (111-1095)	Unk	Surv; RG
						A(H1N1)pdm09	**HRI (221-1637)**	NI (1-6)	RI/HRI (50-751)	NI (1-2)	Clin/OS; Surv; In vitro
						B	NI/RI (2-12)	NI (1)	RI/HRI (15-322)	NI (1-2)	Surv; RG
				H274N		B	Unk	NI (1-3)	NI (1)	NI (1-3)	RG
	276	277	275	E276D		A(H3N2)	RI (15)	HRI (160)	Unk	Unk	RG
	292	293	292	R292K		A(H1N1)	NI (1)	RI (18)	Unk	Unk	RG
						A(H3N2)	HRI (>1000)	NI/RI/HRI (3-134)	RI/HRI (14-719)	Unk	Clin/OS; Surv; RG; In vitro/ZA
						B	HRI (>300)	RI (29)	HRI (502)	Unk	RG
	294	295	294	N294S		A(H1N1)	RI/HRI (40/197)	NI (1/5)	RI (12)	Unk	RG
						A(H1N1)pdm09	HRI (124-208)	NI (3-9)	RI (12)	NI (3)	Surv; RG
						A(H3N2)	HRI (300-1879)	NI (8)	NI (1)	Unk	Clin/OS; RG
						B	RI/HRI (17-61)	NI (1-4)	RI (12-31)	NI (2)	Clin/No; RG
	325	325	325	N325K		A(H1N1)pdm09	RI (19)	NI (8)	NI (3)	Unk	Surv
	358-359[h]	355-356	360	K358/359E		B	NI (2)	NI (2)	HRI (165)	NI (<3)	Surv
	371	368	374	R371K		A(H3N2)	RI (45)	RI (15)	Unk	Unk	RG
						B	HRI (101/407)	RI/HRI (29/145)	HRI (352)	Unk	Surv; RG
	390	386	395	A390E		B	RI (5)	NI (1)	RI (5)	NI (<3)	Surv
	402	398	407	G402S		B	NI (4)	RI (7)	Unk	Unk	Clin/OS; Surv
	429	429	432	D429G		B	NI (1)	NI (1)	RI (41)	NI (<3)	Surv
	442	442	440	S442L [f]		B	NI (2)	NI (2)	RI (7)	NI (1)	Surv
	449	449	449	M449V		B	NI (3)	NI (3)	RI (5)	RI (5)	Surv
	313+427	313+427	313+430	Q313K+I427T		A(H1N1)pmd09	RI (10-43)	NI/RI (3-20)	NI (4)	Unk	Surv
	72+439	74+439	71+439	V72A+H439R[c]		B	NI (1)	NI (1)	RI (19)	RI (1)	Surv
	144+147	144+147	142+145	Y144H+G147R		B	RI (5)	NI (4)	HRI (487)	NI (<3)	Surv
	150+344	150+341	148+343	G150R[f]+K344E		B	NI (1)	NI (1)	RI (7)	NI (1)	Surv
SYNERGISTIC AMINO ACID SUBSTITUTIONS	117	117	115	I117V	E119V	A(H1N1)	-	HRI (391) **3x**	-	-	RG
					N294S+H274Y	A(H1N1)	HRI (336) **3x**	RI (12) **15x**	-	-	RG
				I117M	N294S+H274Y	A(H1N1)	-	RI (11) **9x**	-	-	RG
	119	119	117	E119G	H274Y	A(H1N1)pdm09	-	-	HRI (93433) **298x**	-	Clin/ZA cloning
	148	148	146	T148I	E119V	A(H3N2)	HRI (6154) **22x**	HRI (1050) **350x**	HRI (119) **119x**	HRI (722) **180x**	RG
	151	151	149	D151N	H274Y	A(H1N1)	HRI (799) **2x**	-	HRI (718) **5x**	-	Surv
				D151G	H274Y	A(H1N1)	HRI (1189) **3x**	RI (14) **7x**	HRI (1161) **9x**	-	Surv
	198	199	197	D198N	H274Y	A(H1N1)pdm09	HRI (298) **6x**	-	-	-	Clin/OS
	222	223	221	I222V	H274Y	A(H1N1)	-	-	HRI (893) **3x**	-	RG
						A(H1N1)pdm09	-	-	HRI (1331) **5x**		RG
					N294S+H274Y	A(H1N1)	HRI (300) **3x**	RI (13) **11x**	-	-	RG
				I222K	H274Y	A(H1N1)pdm09	-	NI (7) **6x**	-	-	In vitro
				I222R	H274Y	A(H1N1)pdm09	-	RI (13/16) **13x/16x**	HRI (17347) **26x**	-	Clin/OS; RG
	246	247	245	S246N	H274Y	A(H1N1)pdm09	HRI (7073) **11x**	NI (5) **5x**	HRI (704) **3x**	-	Surv

A(H1N1): former seasonal A(H1N1); Clin: Clinic; Del: deletion; HRI: Highly Reduced Inhibition; NA: Neuraminidase; NI: Normal Inhibition; OS: Oseltamivir; RG: Reverse Genetics; RI: Reduced Inhibition; Surv: Surveillance; Unk: Unknown; x: times; ZA: Zanamivir

[a] Numbering based on the alignment of NA sequences from A/Brisbane/59/2007 (former seasonal A(H1N1)), A/California/07/2009 (A(H1N1)pdm09), A/turkey/Turkey/01/2005 (A(H5N1)), A/Singapore/01/1957 (A(H2N2)), A/Perth/16/2009 (A(H3N2)), A/Hong Kong/1074/1997 (A(H9N2)), A/duck/Jiangsu/1-15/2011 (A(H4N2)), A/Anhui/01/2013 (A(H7N9)), A/Jiangxi-Donghu/346/2013 (A(H10N8)), B/Brisbane/60/2008 (B/Victoria lineage), and B/Bangladesh/3333/2007 (B/Yamagata lineage); [b] Chemiluminescent NA-Star®, fluorescent MUNANA-based and/or colorimetric (fetuin) NA inhibition assays; [c] Not found in the clinical specimen; [d] Not found in the clinical specimen in former seasonal A(H1N1) and A(H3N2) subtypes; [e] Present in the clinical specimen but in only 2.7% of the virus population; [f] Detected as a mixed virus population; [g] Detected as a mixed virus population in A(H1N1)pdm09 subtype; [h] Precise N2 numbering cannot be given as influenza B NA carries an insertion in the alignment compared to A(H3N2) NA.

Amino acid substitutions conferring reduced susceptibility to NA inhibitors (NAIs) *in vitro* are shown separately from those enhancing the level of reduction – synergistic amino acid substitutions. NAI phenotypes follow the IC_{50} fold-change criteria defined by the WHO Expert Working Group on Surveillance of Influenza Antiviral Susceptibility (WHO AVWG) for influenza A (NI: <10-fold, RI: 10 to 100-fold, HRI: >100-fold) and B viruses (NI: <5-fold, RI: 5 to 50-fold, HRI: >50-fold). H274Y amino acid substitution is shown in bold for N1 NA influenza viruses and OS as it is known to cause clinical resistance. After the designation of the synergistic amino acid substitution, it is indicated, in a separated sub-column, the amino acid substitution(s) conferring the reduction in susceptibility. Whenever the synergy involved two amino acid substitutions conferring RI or HRI (e.g. E119G or I222R with H274Y), it was considered as synergistic the substitution with the lowest effect on susceptibility. Only the phenotype on which the synergistic effect was observed is shown, with the level of synergy being highlighted in golden brown and bold.

(Footnotes Table 1.2 cont.)
The section of the table regarding the amino acid substitutions conferring reduced susceptibility was based on the summary table that has been developed by the WHO AVWG [185,186]. NA substitutions conferring NI were only included if their effect is equal or greater than two-fold and confirmed by RG. References for synergistic amino acid substitutions include Choi *et al.* [187], Tamura *et al.* [188], Tamura *et al.* [189], Okomo-Adhiambo *et al.* [190], Ghedin *et al.* [191], Hurt *et al.* [192], Pizzorno *et al.* [193], Huang *et al.* [194], Pizzorno *et al.* [195], and Hurt *et al.* [196].

Several amino acid substitutions in NA have shown to enhance the level of reduced susceptibility conferred by H274Y, alone or in combination with N294S, and E119V - synergistic amino acid substitutions (Table 1.2). These substitutions have essentially no or only little effect on virus susceptibility, with exception of E119G and I222R substitutions in N1pdm09 NA. These confer RI or HRI ((H)RI), but its combined effect with H274Y exceeds the additive effect of their individual contributions. Since both E119G and I222R substitutions have lower effect on susceptibility than H274Y, they were considered the synergistic element of the combination.

Structural basis for reduced susceptibility to NAIs may involve (1) inhibition of the full rotation of residue E276, preventing the formation of the hydrophobic pocket on which the large 6-pentyl ether group of OS and PER binds (H274Y in N1 NA; R292K in N2 NA)[150,197]; (2) conformational changes on the binding-pocket (I222R in N1 NA) [198]; or (3) disruption of the hydrogen bonds established with the carboxylate group (N294S in N1 NA) or the N-acetyl group (D198E in influenza B NA) of the drug [150].

1.5.2.1.2 Hemagglutinin amino acid substitutions

Two amino acid substitutions at position 221 of the HA1 HA of seasonal A(H1N1) viruses (225 in H3 numbering) are currently known to confer reduced NAI susceptibility *in vitro* – D221G (≤100-fold reduction) and D221N (≤10-fold reduction) [199]. The clinical significance of such reductions is, however, still unknown. Residue D221 belongs to the HA RBS and is directly involved in the binding to sialic acid [200].

Several HA1 HA amino acid substitutions have been identified in influenza virus recovered from patients undergoing antiviral therapy, many of whom immunocompromised. Specifically, T198I and S285A substitutions in influenza B HA

(T188I and S271A in H3 numbering); and R142G, Y195F, I239R, V226I and S262N substitutions in H3 HA [201-203]. HA1 HA substitutions have also been selected *in vitro* following passaging under NAI drug pressure, namely: K130E, V132A and K153E substitutions in 2009 H1 pandemic HA (K133E, V135A, and K156E in H3 numbering); and P194L substitution in H3 HA [204]. Only the substitutions selected in cell lines expressing the same type of receptors than human respiratory cells (overexpression of α2,6-linked receptors) were considered. The effect of all above-mentioned substitutions in NAI susceptibility is still unknown but the location of most of them in or near the RBS supports a potential role in susceptibility.

1.5.2.2 Characteristics of Drug-Resistant Variants

Influenza variants resistant or with (H)RI to OS emerge at a low frequency after exposure to the drug. They are detectable in <1 to 4% of OS-treated immunocompetent adults, with the frequency increasing up to 4-8% in the pediatric population, possibly due to prolonged virus shedding [150]. Higher frequencies have been observed in some clinical therapeutic settings, such as in young hospitalized children (16 to 27%) [150], immunocompromised patients under prolonged treatment [202,205,206] and human cases of influenza A(H5N1) virus infection (25%) [207]. OS-resistant or (H)RI variants can emerge as early as 48h after initiating drug treatment [150]. Influenza variants showing (H)RI by PER emerge at similar rates than those observed for OS, varying from <1 to 6.1% among treated adults and children [149]. Reduced susceptibility to ZA following clinical use has been otherwise only rarely observed [49].

Early experience of NAI resistant or H(RI) variants with some of the most common NA amino acid changes, including H274Y in seasonal A(H1N1) and R292K in A(H3N2) backgrounds, showed that such mutant viruses were compromised in their NA enzyme activity and stability and in their infectivity *in vitro* and in animal models [208-210]. Also, their poor infectivity showed to affect virus transmissibility in animal models [210,211]. Resistance or reduced susceptibility to NAIs was therefore considered unlikely to be clinically relevant. However, the emergence of an epidemic H274Y OS–resistant seasonal A(H1N1) variant in late 2007 (detailed below in section 1.5.2.3.1) showed that H274Y mutation can be tolerated in the virus population without fitness loss and may even be

advantageous in certain genetic backgrounds [212]. The presence of fitness-compensatory amino acid substitutions in virus NA has been considered to be on the basis of this fit and transmissible H274Y variant [174]. This compensatory role is currently attributed to NA R222Q, V234M, D344N and D354G substitutions (N1 numbering) [181]. Another set of compensatory substitutions for H274Y mutation might be present in recently circulating 2009 A(H1N1) pandemic viruses. Transmission of H274Y OS-resistant A(H1N1)pdm09 viruses has occurred to a limited extent (detailed in section 1.5.2.3.2), and *in vivo* studies showed that the H274Y mutant virus is at least as virulent as the wild-type A(H1N1)pdm09 virus in mice and ferrets, in spite of the airborne transmission being less efficient [213,214]. NA V241I and N369K substitutions (N1 numbering) may be playing this compensatory role, as evidenced by their ability to restore the detrimental fitness effect of H274Y [181]. A(H3N2) and influenza B (H)RI variants containing NA E119V and NA E119A or H274Y mutations, respectively, showed good *in vitro* and *in vivo* replication and therefore should be closely monitored [174].

1.5.2.3 Resistance Among Circulating Human Influenza Viruses

No pre-existing resistance or reduced susceptibility to NAIs was identified among globally representative human influenza A and B viruses circulating prior to their introduction into clinical practice (from 1996 to 1999) [215]. Moreover, only NA H274Y OS-resistant N1 variants (seasonal and 2009 pandemic A(H1N1)) have been frequently detected to date [216]. N1 H274Y mutation is also know to confer (H)RI by PER *in vitro*, as detailed above in Table 1.2.

1.5.2.3.1 Global emergence of oseltamivir resistance among former seasonal A(H1N1) viruses

During the period from NAI market release (1999) to 2007, NA H274Y OS-resistant seasonal A(H1N1) viruses were detected at a very low global frequency (≤0.5%) [217,218]. Even in Japan, where public health policies supported extensive NAI drug use, OS resistance emerged at a very low frequency (~1.6%) [219]. However, in late 2007, seasonal A(H1N1) viruses harbouring NA H274Y mutation began to circulate at an increasing

frequency in Europe. Many European countries detected the mutation in >20% of their circulating viruses, with France and Norway reporting frequencies above 40% (47% and 68%, respectively) [145]. Moreover, resistant viruses were coming from patients not undergoing OS therapy. NA H274Y OS-resistant viruses spread rapidly to North America and then, by the middle of 2008, were circulating in Asia and the Southern Hemisphere [174]. In addition to spread rapidly, they also outcompeted the sensitive counterparts from circulation, so that, by late 2008, virtually all circulating A(H1N1) viruses were resistant to the drug [220]. This situation continued until seasonal A(H1N1) viruses started to be replaced by the OS-sensitive A(H1N1) virus that caused the 2009 pandemic, with the overall frequency of resistance dropping to negligible levels as seasonal A(H1N1) viruses become extinct [174]. To date, it is still unknown why NA H274Y OS-resistant seasonal A(H1N1) viruses spread globally over their sensitive counterparts, even more in a context of overall low drug use. The presence of fitness-compensatory mutations in virus NA may explain the emergence of a fit and transmissible NA H274Y variant, but it does not explain why OS-resistant viruses surpassed their sensitive counterparts. All fitness-compensatory mutations were also present in the NA H274Y seasonal A(H1N1) viruses that did not spread worldwide (clade 2C OS-resistant viruses) [221,222]. It is possible that amino acid changes located at any of the other segments of influenza virus genome may have contributed for these fitter NA H274Y OS-resistant viruses, highlighting the need of extending genetic analysis to the whole genome.

1.5.2.3.2 Risk of spread of oseltamivir-resistant A(H1N1)pdm09 viruses

To date, the frequency of OS resistance among circulating A(H1N1)pdm09 viruses has remained low on a global scale (<2%) [174]. Earlier NA H274Y OS-resistant viruses were mainly recovered from immunocompromised patients undergoing OS treatment for prolonged periods [145]. But, in 2011, reports from the United Kingdom, Netherlands, USA, and the Asia-Pacific region revealed an overall increase in the proportion of OS-resistant viruses from untreated community patients, suggesting a potential low-level transmission of resistant viruses in the community [145]. Moreover, it was detected for the first time a widespread community cluster of related OS-resistant cases with no known exposure to the drug, in Australia [223]. Three other community clusters involving OS-resistant viruses were recently identified during the 2013/2014 Northern Hemisphere

influenza season. Specifically, two larger clusters in Japan and the USA and a third one in China, smaller and possibly derived from the cluster in Japan [181]. These latter cluster events confirmed that A(H1N1)pdm09 viruses harbouring NA H274Y mutation are able to replicate and transmit between humans as efficiently as sensitive wild-type viruses [125], leading to serious concerns about the occurrence of seasonal epidemics of OS-resistant A(H1N1)pdm09 viruses.

1.5.2.3.3 Reduced susceptibility in A(H3N2) and influenza B viruses

A(H3N2) and influenza B viruses exhibiting (H)RI by NAIs have been rarely reported among circulating viruses. Of note, a limited cluster of influenza B viruses harbouring NA I222V mutation, which confers RI by OS and PER, was detected in the USA during 2010/2011 [224]. Also, the genetic similarity exhibited by NA D198N influenza B viruses circulating in Australia and China during 2014/2015 may indicate a potential low-level transmission of a RI variant in the community setting [125]. NA D198N mutation is known to confer RI by OS, ZA and PER (see Table 1.2 above for detail).

Latest global susceptibility surveillance data indicates that >99% of the human influenza viruses circulating worldwide during 2014/2015 were sensitive to all 4 NAIs [125].

Regarding potentially pandemic viruses, OS resistance (NA H274Y) has been observed in A(H5N1) avian viruses recovered from patients undergoing OS treatment, while A(H7N9) avian viruses have been predominantly sensitive to NAIs [174].

1.6 SURVEILLANCE FOR ANTIVIRAL RESISTANCE

Surveillance activities for tracking the emergence of antiviral resistance in circulating influenza viruses improved significantly after the introduction of NAI drug class. Global and European susceptibility surveillance networks were created, using the existing influenza surveillance systems coordinated by WHO - Global Influenza Surveillance and Response System (GISRS), and the European Centre for Disease Prevention and Control

(ECDC) - European Influenza Surveillance Network (EISN). Globally, the Neuraminidase Inhibitor Susceptibility Network (NISN, 1999-2006) conducted antiviral susceptibility testing on virus isolates provided by the WHO Collaborating Centres (WHO CCs), using a uniquely all-encompassing approach that linked WHO CCs and drug companies with the WHO GISRS [168,225]. The European Surveillance Network for Vigilance against Viral Resistance (VIRGIL, 2004–2008) provided antiviral susceptibility data for more than 30 countries in Europe [225]. Both global and European networks increased antiviral susceptibility testing capacity and enabled the sharing of resources, expertise and data, the standardization of methodologies and a multidisciplinary communication. They also ensured the training and the accomplishment of the individual needs of each country and promoted the rapid detection and timely reporting of drug resistance [225]. Currently, antiviral susceptibility testing is directly carried out at the WHO CCs and at several National Influenza Centres (NICs) of the GIRS network [226]. National laboratories of the ECDC European Reference Laboratory Network for Human Influenza (ERLI-Net), most of which are also WHO NICs, are also testing for antiviral susceptibility. WHO GISRS and ECDC EISN reporting mechanisms are being used for the timely release and sharing of data. Compared to WHO CCs, national laboratories have the capacity to work with a more representative sample and to deliver the results in a more timely manner, which is critical for the early detection of resistant variants in either the community or clinical management [226]. In fact, Denmark reported the first case of OS resistance in A(H1N1)pdm09 subtype, three months after the emergence of the new virus [227]. Practical guidelines for national laboratories testing influenza virus susceptibility were recently provided by the expert working groups that were established under the umbrella of the WHO GISRS and the ECDC EISN – WHO AVWG and ECDC Antiviral Susceptibility Task Group [228,229]. Both groups have also the role of providing advice on the system surveillance strategies for influenza antiviral susceptibility [183,229]. Due to the overall resistance to M2 inhibitor drugs since 2009, M2 inhibitor susceptibility testing is no longer considered a priority [229].

1.6.1 *Clinical and Laboratory Surveillance*

Drug resistance can be defined (1) clinically, when a treated person is refractory to drug treatment or occurs person-to-person transmission of a virus that is not sensitive to drug

treatment; (2) phenotypically, by measuring the drug susceptibility of a virus isolate in a model system, with resistance implying a measurable alteration in a virus property; or (3) genetically, by a change in the virus genome associated with a measurable phenotypic loss of susceptibility and/or clinical resistance [161].

Since the molecular basis of M2 inhibitor resistance is well established (see section 1.5.1.1 for detail), genotypic evaluation of virus susceptibility through molecular-based assays is sufficient to monitor the susceptibility of circulating influenza virus to this antiviral drug class. Laboratory surveillance of influenza virus susceptibility to NAIs implies otherwise both phenotypic and genotypic evaluations [229], with NA inhibition assays constituting the gold standard for phenotypic susceptibility testing [228]. Only the presence of NA H274Y mutation in N1 NA influenza viruses can be exclusively analysed through genotypic testing – single molecular marker of NAI resistance.

1.6.2 Why is so important monitoring influenza antiviral susceptibility?

Influenza antiviral susceptibility monitoring activities are important for individual patient treatment. Monitoring the emergence of viruses resistant or with (H)RI in hospitalized patients allows to make decisions on possible changes to antiviral treatment. Moreover, information on the frequency of circulating influenza viruses with natural resistance or (H)RI in the community setting is essential for making first choice antiviral drug treatment decisions and to advise public health policy-makers on antiviral use or strategic stockpiling [229]. Monitoring activities are also important for early detection of resistant or (H)RI variants, particularly those with good viral fitness that can be readily transmissible and become dominant among circulating viruses [229].

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CHAPTER 2

STUDY DESCRIPTION

2 STUDY DESCRIPTION

2.1 STUDY GROUNDS

Antiviral resistance in influenza may not only emerge during patient treatment or prophylaxis but also spontaneously in the absence of drug-selective pressure [1]. The potential risk of emergence of influenza variants naturally resistant to antiviral drugs, associated with the rapid and unpredictable nature of influenza virus evolution, highlights the need for active and sustained surveillance for drug resistance in circulating influenza viruses. National surveillance activities are important for (1) the early detection of drug-resistant variants in the community or in clinical management [2]; and (2) improving the coverage and representativeness of the antiviral susceptibility data collected on a global scale, in which are based the guidelines and/or recommendations on the use of influenza antivirals and on their strategic stockpiling as part of pandemic preparedness. Implementing antiviral susceptibility testing in Portugal was therefore essential, comprising the first objective of this research project. We purposed to evaluate and monitor the susceptibility of human influenza viruses from all different types and subtypes circulating at national level since 2004/2005, expecting to cover both community and hospital settings. Influenza viruses were recovered from community patients presenting with influenza-like illness (ILI) to healthcare systems covered by the National Influenza Surveillance Programme (2004/2005 to 2008/2009), and from patients either admitted to the emergency unit or hospitalized at the Hospital Curry Cabral (HCC, Centro Hospitalar de Lisboa Central, EPE) (from 2009 onwards). HCC was the national reference hospital for both laboratory diagnosis and hospitalization during 2009 A(H1N1) influenza pandemic and is the national reference hospital for rapid laboratory confirmation of suspected cases of clinical resistance to influenza antiviral drugs.

The efficient fitness and transmissibility of the neuraminidase (NA) H275Y oseltamivir (OS)-resistant former seasonal A(H1N1) variant that emerged in late 2007, have been attributed to the presence of additional amino acid substitutions in NA – potentially R222Q, V234M, D344N and D354G (N1 numbering) [3,4]. These substitutions counteracted the detrimental effect of the H275Y substitution on NA enzyme activity and virus replicative properties [5-8]. However, its presence cannot explain why NA H275Y former

seasonal A(H1N1) viruses spread over their OS-sensitive counterparts, outcompeting them from circulation. They were also found in NA H275Y viruses from a different clade (clade 2C) than the one that spread worldwide, fixing NA H275Y in A(H1N1) virus population (clade 2B) [9,10]. Based on this, it has been suggested that NA H275Y substitution may have occurred coincidentally with other advantageous mutation(s) located elsewhere in the viral genome and hitchhiked to fixation along with them (mutation hitchhiking) [10,11]. With the aim of contributing at a better understanding of which mutations in viral genome may have played a role in the enhanced viral fitness of NA H275Y OS-resistant viruses, we purposed to determine the complete genome of NA H275Y OS-resistant former seasonal A(H1N1) viruses circulating in Portugal and to identify and characterize their specific amino acid substitutions. We further proposed to extend this whole-genome analysis to all other influenza viruses identified as resistant or with decreased susceptibility to neuraminidase inhibitor (NAI) drugs, in order to assess potential risks of spread, by looking for mutations that may enhance virus fitness.

NAIs are presently the only approved antivirals effective against circulating human influenza viruses, targeting the virus NA protein that is also under selective pressure (SP) from the host's antibody-mediated immune response (second major surface antigen) [12]. SP may therefore play an important role in the molecular dynamics underlying the emergence and spread of influenza variants resistant or with (highly) reduced inhibition ((H)RI) to NAIs *in vitro*. Over the last few years, several research studies have estimated both global and site-specific SP acting on NA, but only a few of them investigated the SP acting on the sites associated with NAI resistance or (H)RI [13-19]. Furthermore, only a limited number of the sites currently associated with (H)RI was considered in the different analyses and none of the studies focused on type B influenza NA. Hence, we purposed to study the SP acting on the influenza NA of all virus subtypes and lineages circulating among humans (former seasonal A(H1N1), A(H3N2), B/Victoria and B/Yamagata-lineage, and A(H1N1)pdm09), focusing the analysis on the sites associated with NAI resistance or (H)RI and on further sites contacting directly or indirectly with the drug (active site). NA sequence datasets were constructed using all potentially complete NA sequences of influenza viruses circulating worldwide available at the Global Initiative on Sharing All Influenza Data (GISAID) EpiFlu™ and the National Center for Biotechnology Information (NCBI) Influenza Virus Resource databases. Unpublished sequences from viruses circulating in Portugal were further added to the datasets. We

further proposed to investigate for the first time the impact of NAI introduction into clinic (1999) and its increased use during 2009 A(H1N1) pandemic on both global and site-specific SP acting on influenza NA. For that, sequences were split into three temporal sub-datasets defined by the occurrence of such events: (1) before 1999 (no NAI use); (2) 1999-2008 (low overall NAI use); and (3) from 2009 onwards (increased NAI use). A(H1N1)pdm09 NA sequences were exceptionally split into two temporal sub-datasets defined by the end of the pandemic period (10/08/2010), after which occurred a decrease in NAI use [20].

In summary, this PhD project followed three main lines of research: (1) antiviral susceptibility testing; (2) whole-genome sequencing; and, (3) selective pressure acting on influenza NA.

2.2 AIM AND OBJECTIVES

This research project aimed at disclosing the susceptibility of human influenza viruses circulating in Portugal to nationally approved antivirals; and at improving the knowledge on the evolutionary dynamics underlying the emergence and/or spread of influenza variants resistant or with decreased susceptibility to NAI antiviral drugs.

It comprised three general objectives, with the first and third ones further including four and two specific objectives, respectively.

1st General Objective

To evaluate and monitor the susceptibility of human influenza viruses circulating in Portugal from 2004/2005 onwards, in both community and hospital settings, to the three antivirals approved nationally for clinical use – amantadine (M2 protein inhibitor), oseltamivir (OS) and zanamivir (ZA) (NAIs).

Specific Objectives

1(a) To establish a technological platform for phenotypic and/or genotypic evaluation of influenza virus susceptibility to M2 protein inhibitors and both NAIs OS and ZA;

1(b) To determine the antiviral susceptibility profile of human influenza viruses from all

different types and subtypes (former seasonal A(H1N1), A(H3N2), B and A(H1N1)pdm09);

1(c) To analyze the relationship between reduced NAI susceptibility phenotypes and genotypic background and, whenever possible, between these and patient clinical data;

1(d) To detect variations or trends in the natural *in vitro* susceptibility of circulating viruses to NAIs over time (baseline phenotypic drug susceptibility).

2nd General Objective

To identify and characterize the amino acid substitutions specific of the genome of influenza viruses resistant or with decreased susceptibility to OS and/or ZA.

3rd General Objective

To study the selective pressure (SP) acting on the NA gene of worldwide circulating viruses from all human influenza subtypes and lineages - former seasonal A(H1N1), A(H3N2), B/Victoria-lineage, B/Yamagata-lineage and A(H1N1)pdm09.

Specific objectives

3(a) To estimate the SP acting on the sites associated with NAI resistance or (H)RI *in vitro* and on further sites contacting directly or indirectly with the drug (active site);

3(b) To investigate the impact of NAI introduction into clinic (1999) and its increased use during 2009 A(H1N1) pandemic on the global and site-specific SP acting on human influenza NA.

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CHAPTER 3

MATERIAL AND METHODS

3 MATERIAL AND METHODS

3.1 MATERIAL

This section describes the key biological materials, chemical commercial products and influenza virus sequences available in public-access databases used in this study. All remaining materials are indicated within the description of the procedure in which they were used, in the following methods section (section 3.2).

3.1.1 Influenza Virus Isolates and Influenza-Positive Clinical Specimens

All influenza viruses isolated at the National Influenza Centre (NIC), Instituto Nacional de Saúde Doutor Ricardo Jorge (INSA), IP, between 2004/2005 and 2008/2009 influenza seasons[A], were selected for antiviral susceptibility testing. This involved a total of 421 virus isolates (102 former seasonal A(H1N1); 187 A(H3N2); and 132 influenza B), recovered from more than 30% of all influenza-positive clinical specimens detected during those seasons (Table 3.1). Clinical specimens (mostly nasopharyngeal and oropharyngeal swabs) were collected from influenza-like illness (ILI) patients who consulted with a sentinel medical practitioner or attended an emergency unit participating in the National Influenza Surveillance Programme. The accompanying notification forms included information on antiviral prescription and/or exposure (either by direct use or contact with a patient on therapy) since 2005/2006.

In 2009, the source for influenza viruses changed, with the Hospital Curry Cabral (HCC, Centro Hospitalar de Lisboa Central, EPE), providing influenza-positive clinical specimens (mostly nasopharyngeal and oropharyngeal swabs) from patients either admitted to the emergency unit or hospitalized. This change implied performing viral isolation before antiviral susceptibility testing. All positive clinical specimens collected until the 2012/2013 influenza season were considered for study and used for viral isolation. The only exception were those testing positive for the new pandemic virus during the 2009 pandemic period[B] that, due to the very high number of virus detections (n=577), were selected using 5 main criteria. Specifically: (1) collected from patients

[A] By convention, the influenza season in Europe is considered to begin with week 40 of any given year (early October), extending through week 20 of the following year (mid-May) [1].

[B] Period from 11th June 2009 to 9th August 2010 - WHO pandemic alert Phase 6.

suspected of harbouring drug-resistant virus (clinical suspicion of drug-resistance); (2) known association to antiviral drug use; (3) collected from high-risk group patients; (4) collected from deceased patients or patients with severe disease; and (5) distribution over time and space (data representativeness). Overall, 321 clinical specimens positive for influenza virus (2 former seasonal A(H1N1); 38 A(H3N2); 26 influenza B; and 255 A(H1N1)pdm09), were selected for studying antiviral susceptibility (Table 3.1).
Effective study sample is described in chapter 4.

Table 3.1 Number of influenza virus isolates and influenza-positive clinical specimens selected for antiviral susceptibility testing.

INFLUENZA VIRUS ISOLATES (National Influenza Centre)

Influenza season	Influenza virus type/subtype	Number [a]
2004/2005	seasonal A(H1N1)	6 (100%)
	A(H3N2)	89 (25.3%)
	B	35 (48.6%)
	TOTAL	130 (30.2%)
2005/2006	seasonal A(H1N1)	46 (65.7%)
	A(H3N2)	1 (16.7%)
	B	49 (55.1%)
	TOTAL	96 (58.9%)
2006/2007	seasonal A(H1N1)	-
	A(H3N2)	76 (25.2%)
	B	2 (66.7%)
	TOTAL	78 (25.7%)
2007/2008	seasonal A(H1N1)	29 (54.7%)
	A(H3N2)	-
	B	45 (66.2%)
	TOTAL	74 (61.2%)
2008/2009	seasonal A(H1N1)	21 (32.8%)
	A(H3N2)	21 (8.4%)
	B	1 (16.7%)
	TOTAL	43 (13.4%)
TOTAL	seasonal A(H1N1)	102 (52.8%)
	A(H3N2)	187 (20.5%)
	B	132 (55.5%)
	TOTAL	421 (31.4%)

INFLUENZA-POSITIVE CLINICAL SPECIMENS (Hospital Curry Cabral)

Pandemic period / Influenza season	Influenza virus type/subtype	Number
2009 pandemic period [b]	seasonal A(H1N1)	2
	A(H3N2)	5
	B	10
	A(H1N1)pdm09	163 [c]
	TOTAL	180
2010/2011	seasonal A(H1N1)	-
	A(H3N2)	2
	B	5
	A(H1N1)pdm09	69
	TOTAL	76
2011/2012	seasonal A(H1N1)	-
	A(H3N2)	30
	B	2
	A(H1N1)pdm09	-
	TOTAL	32
2012/2013	seasonal A(H1N1)	-
	A(H3N2)	1
	B	9
	A(H1N1)pdm09	23
	TOTAL	33
TOTAL	seasonal A(H1N1)	2
	A(H3N2)	38
	B	26
	A(H1N1)pdm09	255
	TOTAL	321

seasonal A(H1N1): former seasonal A(H1N1)

[a] In brackets is indicated from which percentage of the total influenza type/subtype-matched positive clinical specimens, the virus isolates were recovered; [b] Period from 11th June 2009 to 9th August 2010 - WHO pandemic alert Phase 6; [c] Represents 28.2% (163/577) of the total clinical specimens positive for A(H1N1)pdm09 virus during that period.

Among all virus isolates and clinical specimens selected for study, 33 (14 isolates; 19 clinical specimens) were from cases of influenza virus infection associated with antiviral

use, with 5 specimens (3+2) belonging to the same two cases – 30 cases in total (29 oseltamivir (OS); 1 zanamivir (ZA)). Nevertheless, only 10 of the clinical specimens were effectively associated with drug use, having been collected during and particularly after antiviral therapy. Four of these specimens were received with a high level of suspicion of clinical resistance to OS (A(H1N1)pdm09 virus infection). Detailed information can be found in Table 3.2.

Table 3.2 Epidemiological, clinical and laboratorial information concerning the 4 cases of influenza A(H1N1)pdm09 virus infection received with clinical suspicion of oseltamivir resistance.

	Case	**1** (pandemic period)	**2** (2010/2011)	**3** (2010/2011)	**4** (2010/2011)
Patient	Age (years)	15	1	27	61
	Gender	Male	Male	Female	Male
	Underlying risk conditions	NR	Premature bronchopulmonary dysplasia	HIV+; Pregnant	NR
Clinical disease		NR	Severe	Severe, loss of the fetus	Severe
Evidence of failure		NR	Positive virus detection after 15 days of treatment	NR	Positive virus detection after 10 days of treatment
Time from the start of AT to specimen collection		Starting date not provided	20 days	Starting date not provided	>=10 days
Clinical outcome		NR	NR	Deceased	NR
Other observations		-		Initiated treatment with ZA; from Guinea, Africa	
N1 NA H275Y screening result [a]		Not performed	Not present	Present (Ct=13)	Present as quasi-species (Ct=27)

AT: Antiviral therapy; Ct: threshold cycle (real-time PCR assay); HIV+: Positive for HIV infection; NR: Not referred; ZA: Zanamivir

[a] Rapid initial screening performed at the Hospital Curry Cabral.

Reference influenza viruses, kindly provided by Dr Alan Hay (National Institute for Medical Research (NIMR), Mill Hill, London, United Kingdom (UK)), and influenza viruses with known antiviral susceptibility profile circulating in Portugal were used for (1) the establishment and quality control of phenotypic neuraminidase (NA) inhibition assays (detailed in section 3.2.1.3); and, (2) optimization of the one-step RT-PCR protocol for whole genome sequencing of former seasonal A(H1N1), A(H3N2) and influenza B viruses.

3.1.2 MDCK and MDCK-SIAT1 Cell Lines

MDCK (Madin-Darby canine kidney) cells were kindly provided by Dr Alan Hay (NIMR, Mill Hill, London, UK), and MDCK-SIAT1 cells (MDCK cells transfected with cDNA of

human β-galactoside α2,6-sialyltransferase (SIAT1)) were obtained from the European Collection of Authenticated Cell Cultures (ECACC) (Public Health England, Salisbury, UK). MDCK cells were used to propagate influenza viruses from 2004/2005 to 2008/2009 seasons that were already MDCK cell-adapted, when it was necessary to increase virus titre or virus stock volume. MDCK-SIAT1 cells were used for isolation of influenza viruses from clinical specimens from the 2009 pandemic period to the 2012/2013 influenza season. Both cell lines were used for producing the virus control panels.

3.1.3 Guinea Pig Red Blood Cells

Guinea pig red blood cells (RBCs) were obtained from the Instituto de Higiene e Medicina Tropical (IHMT)(Lisbon, Portugal), having been collected into Alsever's solution (anti-clotting agent). These RBCs were used for detection and titration of influenza viruses by haemagglutination assay.

3.1.4 Neuraminidase Inhibitors – Oseltamivir and Zanamivir

Oseltamivir carboxylate, the active metabolite of the prodrug oseltamivir phosphate, was kindly provided by F. Hoffmann-La Roche Ltd (Basel, Switzerland). For simplicity, oseltamivir carboxylate is abbreviated as oseltamivir (OS) throughout the text. Zanamivir (ZA) was kindly provided by GlaxoSmithKline (Hertfordshire, UK). Both antivirals were received through a Material Transfer Agreement.
OS and ZA were used in the fluorescent and chemiluminescent NA inhibition assays carried out for phenotypic evaluation of influenza virus susceptibility to neuraminidase inhibitor (NAI) drugs.

3.1.5 Light-Emitting Phenotypic Substrates

MUNANA (2′ 2′-(4-Methylumbelliferyl)-α-D-N-acetylneuraminic acid sodium salt hydrate) substrate was obtained from Sigma-Aldrich (Schnelldorf, Germany), while NA-Star® substrate was supplied in the commercial kit NA-Star® Influenza Neuraminidase Inhibitor Resistance Detection, distributed exclusively by Applied Biosystems and here

obtained from Applera Internacional Inc (Nieuwerkerk aan den Ijssel, The Netherlands). MUNANA and NA-Star® are, respectively, the fluorescent and chemiluminescent substrates of NA inhibition assays.

3.1.6 Neuraminidase Sequences of Worldwide Circulating Human Influenza Viruses

NA gene sequences of worldwide circulating viruses from all human influenza subtypes or lineages, were retrieved from the Global Initiative on Sharing All Influenza Data (GISAID) EpiFlu™ Database (http://platform.gisaid.org), and the National Center for Biotechnology Information (NCBI) Influenza Virus Resource Database (https://www.ncbi.nlm.nih.gov/genomes/FLU). All available sequences with a potentially complete coding region (1410 nucleotides (nts), former seasonal A(H1N1); 1407nts, A(H3N2) and A(H1N1)pdm09; 1398nts, influenza B) were collected for study. To these sequences were further added unpublished sequences of viruses circulating in Portugal since 2000/2001.

Table 3.3 Number and time period covered by the neuraminidase gene sequences of worldwide circulating human influenza viruses collected for study.

Influenza virus (sub)type or lineage	Temporal sub-dataset	Accession date[a]	Number				Time period covered (years)
			GISAID EpiFlu™ Database	NCBI Influenza Virus Resource Database	Portugal	*TOTAL*	
Former seasonal A(H1N1)	1 Before 1999	04/01/2012 [b]	198	215	0	*413*	1918 - 1998 (81)
	2 1999-2008		2022	1408	54	*3484*	1999 - 2008 (10)
	3 From 2009 onwards		406	400	12	*818*	2009 - 2011 (3)
	TOTAL	-	2626	2023	66	*4715*	1918 - 2011 (94)
A(H3N2)	1 Before 1999	07/04/2013	1012	978	0	*1990*	1968 - 1998 (31)
	2 1999-2008		3386	2987	71	*6444*	1999 - 2008 (10)
	3 From 2009 onwards		5055	1271	2	*6328*	2009 - 2013 (5)
	TOTAL	-	9453	5236	73	*14762*	1968 - 2013 (46)
B/Victoria	1 Before 1999	11/10/2013	75	-	0	*75*	1972 - 1998 (27)
	2 1999-2008		478	-	4	*482*	1999 - 2008 (10)
	3 From 2009 onwards		2227	-	1	*2228*	2009 - 2013 (5)
	TOTAL	-	2780	-	5	*2785*	1972 - 2013 (42)
B/Yamagata	1 Before 1999	11/10/2013	101	-	0	*101*	1973 - 1998 (26)
	2 1999-2008		533	-	20	*553*	1999 - 2008 (10)
	3 From 2009 onwards		1503	-	0	*1503*	2009 - 2013 (5)
	TOTAL	-	2137	-	20	*2157*	1973 - 2013 (41)
B[c]	1 Before 1999	11/10/2013	-	201	-	-	1940 - 1998 (59)
	2 1999-2008		-	1183	-	-	1999 - 2008 (10)
	3 From 2009 onwards		-	1388	-	-	2009 - 2013 (5)
	TOTAL	-	-	2772	-	-	1940 - 2013 (74)
A(H1N1)pdm09	1 (Pre-)pandemic period[d]	30/04/2013	5860	5808	44	*11712*	2009 - 09/08/2010 (1.8)
	2 Post-pandemic period		2027	894	13	*2934*	10/08/2010 - 2013 (2.8)
	TOTAL	-	7887	6702	57	*14646*	2009 - 2013 (5)

(Footnotes Table 3.3)

[a] Date of accession to databases (i.e. date in which the sequences were retrieved); [b] Re-search in early 2013 revealed very few new sequences available, avoiding the need of updating preliminary sequence sub-datasets and re-performing sequence treatment and data analysis; [c] B lineage information was not available for most neuraminidase (NA) sequences available at NCBI database; [d] Pre-pandemic (late March to 10th June 2009) or pandemic (11th June 2009 to 9th August 2010) period.

Sequences were grouped according to the temporal split here proposed to investigate the impact of the different contexts of NA inhibitor (NAI) drug use in the selective pressure acting on NA (three or two time periods). When completely treated (see section 3.2.3.1), the sequences were merged to form total datasets.

These sequences were used to study the selective pressure (SP) acting on the NA gene of all influenza virus subtypes or lineages circulating among humans. The temporal split purposed in this study, into three (before 1999, 1999-2008, from 2009 onwards) or two ((pre-)pandemic[C] and post-pandemic periods; A(H1N1)pdm09) time periods according to the differences in NAI global use (detailed in chapter 2), was taken into consideration for retrieving and handling NA sequences. Only when completely treated (see section 3.2.3.2), the sequences were merged to form total datasets. Detailed information on the number and time period covered by NA sequences can be found in Table 3.3.

3.2 METHODS

3.2.1 Laboratory Procedures

3.2.1.1 Culture of MDCK and MDCK-SIAT1 Cells

3.2.1.1.1 Recovery of frozen cells

Low-passage MDCK and MDCK-SIAT1 cells were recovered from liquid nitrogen temperature (-196°C), following the recommendations of the ECACC [2]. Briefly, the frozen cell ampoule was transferred in ice to a 37°C water bath for rapid thawing (1-2 minutes). Once thawed, the whole content of the ampoule was slowly transferred to a 15ml conical centrifuge tube containing 5ml of pre-warmed base medium. For MDCK cells, this medium contained 1x Eagle's minimum essential medium (MEM; Gibco, Paisley, UK), 2mM L-glutamine (Gibco) and 1x non-essential amino acids (NEAA; Gibco); while for MDCK-SIAT1 cells, it contained 1x Dulbecco's modified Eagle's medium (DMEM; Gibco)

[C] Pre-pandemic (late March to 10th June 2009) or pandemic (11th June 2009 to 9th August 2010) period.

supplemented with 2mM L-glutamine, 1x NEAA and 24mM HEPES (Gibco). Cell suspension was centrifuged at 300xg for 5 minutes (min) for removing the cryoprotectant and the cell pellet was resuspended in 2ml of fresh complete growth medium prepared for seeding the cells in tissue culture flaks (7ml for 25cm^2 flasks; 15ml for 75cm^2 flasks). Complete growth medium included pre-warmed base medium supplemented with 8% (v/v) heat-inactivated fetal bovine serum (FBS, Gibco) for MDCK cells and with 10% (v/v) FBS and 1 mg/ml antibiotic G418 sulfate (Geneticin®, Gibco) for MDCK-SIAT1 cells. Cells were then seeded by adding the cell suspension to a 25cm^2 or 75cm^2 flask containing the remaining volume of the growth medium, followed by incubation at 37ºC until achieving total confluence (generally 24h for 25cm^2 flasks and 48h for 75cm^2 flasks).

3.2.1.1.2 Cell maintenance

Cells were transferred to maintenance medium when completely confluent. Growth medium was discarded from the flask and the cell monolayer was washed twice with pre-warmed phosphate buffered saline (PBS) without Ca2+/Mg2+ (Gibco). Pre-warmed maintenance medium was added to the flask, adding 7ml to 25cm^2 flasks and 15ml to 75cm^2 flasks. For MDCK cells, this medium consisted in the base medium, while for MDCK-SIAT1 cells it consisted in the base medium further supplemented with 1 mg/ml G418 sulfate. Cells were then incubated at 30ºC during a maximum of 5 days.

3.2.1.1.3 Cell passage (subculture)

Cells were passaged within the 5 days of maintenance, preferably at day 2 to 4, to prevent the culture dying and to have cultures for viral isolation and/or propagation. Maintenance medium was discarded from the flask and the cell monolayer was washed twice with pre-warmed PBS. After that, pre-warmed 0.05% (v/v) trypsin–EDTA (Gibco) was added to the flask, adding the necessary volume to cover the entire monolayer (2ml to 25cm^2 flasks; 3ml to 75cm^2 flasks). Trypsin-EDTA was maintained in direct contact with the cell monolayer at room temperature, until the monolayer become opaque (1-2min). All volume of trypsin-EDTA was then removed and the flask was incubated at

37ºC until all cells have become detached. During this incubation period, the side of the flask containing the cell monolayer was gently tapping every few min for checking cell detachment. Once complete cell detachment was observed, 4.5ml (25cm^2 flasks) or 9ml (75cm^2 flasks) of pre-warmed base medium were pipetted directly against the side of the flask that contained the cell monolayer, followed by successive up and down pipetting's for mixing and for breaking-up any cell clumps. Cell suspension was then supplemented with 10% (v/v) FBS (0.5ml for 25cm^2 flasks; 1ml for 75cm^2 flasks), mixing gently for reducing air bubble formation that could cause cell wall disruption.

MDCK cell suspension was directly diluted in complete growth medium for producing new maintenance cultures and in complete growth medium further supplemented with 1x penicillin-streptomycin-neomycin antibiotic mixture (PSN; Gibco) for preparing tubes (round, 7ml) and/or flasks (25cm^2 or 75cm^2) for viral isolation and/or propagation. A split ratio of 1:5 was used for obtaining flasks with 80–90% confluence (confluence suitable for viral inoculation) in 48 hours (1.4ml suspension in 5.6ml medium for 25cm^2 flasks; 3ml suspension in 12ml medium for 75cm^2 flasks). A split ratio of 1:3 was used for getting tubes with the same suitable confluence in 24 hours (1ml suspension per tube).

MDCK-SIAT1 was a more recent cell line and thereby no pre-determined straightforward dilutions were used. Viable cell concentration was estimated for each MDCK-SIAT1 suspension after counting the viable cells with a haemocytometer (detailed in section 3.2.1.1.4), in order to determine the required volume of cell suspension to be used for obtaining the suitable confluence (80–90%) on the desired day. For flasks, this volume was the area of the flask (25 or 75) multiplied by a time-variable factor (for 48 hours: 2 for 25cm^2 flasks; 4 for 75cm^2 flasks), and then divided by the viable cell concentration; while for tubes, it was the cell seeding density (30x10^4 cell/ml for 24 hours) divided by the viable cell concentration. Cell suspension was then diluted in complete growth medium for producing new maintenance cultures and in complete growth medium containing 2.5µg/ml Fungizone® antimycotic (Gibco) and 1x PSN instead of 1 mg/ml G418 sulfate for preparing tubes and/or flasks for viral isolation and/or propagation. Total dilution volumes were 1ml, 7ml and 15ml for, respectively, tubes, 25cm^2 and 75cm^2 flasks.

Both cell lines were continually subcultured until reaching 25 consecutive passages or 3 months of use, which are the recommended limits to guarantee their susceptibility to respiratory viruses [3]. A new frozen ampoule of low-passage cells was recovered whenever one of the limits was reached.

3.2.1.1.4 Cell counting using a haemocytometer

Cell counting using a haemocytometer was performed according to the recommendations of the ECACC [2]. Briefly, a coverslip was moisten with water and affixed to the haemocytometer. Equal volumes (20µl) of 0.4% trypan blue stain (Gibco) and cell suspension were gently mixed and approximately 10µl of the mixture was carefully pipetted at the edge of the coverslip, flowing underneath. The haemocytometer grid was then visualized under an inverted microscope (using phase contrast) for counting the viable cells, which appeared colourless and bright (refractile) for not incorporating the trypan blue (dead cells were blue and non-refractile). Viable cell counting was performed in four different squares of the grid (1 square=1mm^2 section), including cells in the middle of square and cells that overlap edge on two sides. Cells overlapping edge on the other two sides were excluded. Viable cell concentration was estimated using the equation below:

$$\underset{\text{(live cells per millilitre)}}{\text{Viable cell concentration}} = \frac{\text{Number live cells counted}}{\underset{\text{(number squares counted)}}{4}} \times \underset{\text{(dilution factor)}}{2} \times \underset{\text{(square grid volume)}}{10^4}$$

3.2.1.2 Influenza Virus Isolation and Propagation in Cell Culture

3.2.1.2.1 Virus isolation and propagation in MDCK and MDCK-SIAT1 cells

Cell culture tubes containing MDCK or MDCK-SIAT1 monolayers were used for influenza virus isolation and/or propagation. Growth medium was discarded from the tubes and cell monolayer was washed twice with pre-warmed base medium (composition detailed in section 3.2.1.1.1). For virus isolation, the cell monolayer was inoculated with 250µl of a mixture containing 200µl of clinical specimen, 25µg/ml Fungizone® and 10X PSN, pre-incubated at room temperature during 30min; while for virus propagation, it was inoculated with 200µl of virus isolate. One or two tubes were additionally inoculated with 200µl of base medium and used as controls. Once inoculated, tubes were incubated at room temperature during 30min to allow virus adsorption, followed by addition of 1ml of infection medium. This consisted in base medium further supplemented with 1mM HEPES, 2.5µg/ml Fungizone®, 1x PSN and 3µg/ml TPCK-treated trypsin (Worthington, Lakewood, NJ, United States of America (USA)). Tubes were then incubated at 35ºC

(influenza A viruses) or 32ºC (influenza B viruses) and checked daily on the microscope for cytopathic effect (CPE), during a maximum of 7 days. Influenza virus-induced CPE is non-characteristic, being assessed by the amount of swollen cells detached from the growing surface. Some influenza viruses may even replicate without producing CPE in the cell monolayer. Culture supernatants were tested by haemagglutination assay (see section 3.2.1.2.2) to confirm or assess the presence of influenza virus when, respectively, 75-100% CPE was observed or no CPE was observed after 7 days of inoculation. A centrifugation step at 5000rpm for 10min was performed at the end, before storing virus isolates, to remove excess cells.

Virus propagation continued in 25cm^2 or 75cm^2 flasks of confluent cells when producing virus controls, following the same procedure above described but using different volumes of inoculum and infection medium. Specifically, 1ml inoculum and 6ml infection medium for 25cm^2 flasks; 2ml inoculum and 13ml infection medium for 75cm^2 flasks.

3.2.1.2.2 Haemagglutination assay

Haemagglutination assay was used to assess or confirm the presence of influenza virus and, when present, to determine virus titre. The assay was performed according to the World Health Organization (WHO) recommendations [3], using a 96-well U-bottom microtitre plate and 0.75% guinea pig RBCs (see preparation in section 3.2.1.2.3). The plate was positioned so that the 12-well side was horizontal (columns 1 to 12, left to right) and the 8-well side was vertical (rows A to H, top to bottom). PBS was added to wells 2 to 12 of each row, adding 50µl per well. Then, 100µl of each cell culture supernatant was added to the first well of rows A–G (up to 7 different supernatants tested per plate). RBCs controls were prepared by adding 50µl PBS to two wells of row H. Two-fold serial dilutions of culture supernatants were performed throughout the plate, transferring 50µl from the first to successive wells of each row, discarding the final 50µl. After this dilution step, 50µl of 0.75% guinea pig RBCs were added to each well. The plate was carefully agitated by gentle tapping, covered and incubated at room temperature during 45min. RBCs controls were then checked for complete settling of the cells and, when validated, the titration end-point of culture supernatants was determined, corresponding to the highest dilution that still caused complete agglutination (RBCs in suspension, completely agglutinated by the hemagglutinin (HA) proteins of the virus).

The virus titre is the reciprocal of this end-point dilution (e.g. 1:256 dilution, virus titre=256), being given in haemagglutination units (haemagglutination unit = amount of virus needed to agglutinate an equal volume of a RBCs suspension). Non-agglutinated guinea pig RBCs (halo or circle of settled cells on the bottom of the well), indicated that no virus was present or that the virus was not present in a sufficient quantity for causing agglutination.

3.2.1.2.3 Preparation of guinea pig red blood cells 0.75% suspension

Guinea pig RBCs suspension in Alsever's was transferred to a conical centrifuge tube (5ml to a 15ml tube), filling the remaining volume of the tube (10ml) with PBS. The tube was gently mixed by several slowly inversions and then centrifuged at 1800rpm for 10min. The supernatant and the 'buffy' layer of white cells were aspirated and 10ml of PBS were added to the tube. RBCs pellet was gently resuspended by slowly inverting the tube several times. A new centrifugation step at 1800rpm for 10min was performed, followed by aspiration of the supernatant. These washing cycling steps (add PBS – resuspend – centrifuge – aspirate supernatant) were continually performed until the supernatant was clear (indicative of no cell lysis; usually more two times). When that was verified, it was removed as much supernatant as possible and the washed RBCs were diluted for a final concentration of 0.75% (v/v).

3.2.1.3 Phenotypic Neuraminidase Inhibition Assays

NA inhibition assays required the use of a standard NA activity for each virus. To determine the standard virus dose to use in each assay (virus dilution), virus isolates were titrated based on their NA activity (NA activity assay).

The panel of virus controls used for implementation and validation of NA activity and inhibition assays included initially one former seasonal A(H1N1) virus (A/Texas/36/1991 or A/Lisboa/18/2008), one A(H3N2) virus (A/Wisconsin/67/2005 or A/Lisboa/01/2009), and one influenza B virus (B/Memphis/20/1991 or B/Lisboa/09/2008), all susceptible to OS and ZA. In 2009 and 2011, were added to the panel one A(H1N1)pdm09 virus susceptible to both NAIs (A/Portugal/1/2009) and one

A(H1N1)pdm09 virus resistant to OS (A/Portugal/03/2011; NA H275Y), respectively. All virus controls were tested in 10 to 15 independent assays before being used for validation.

The FLUOstar OPTIMA fluorometer (BMG Labtech, Madrid, Spain), equipped with a dedicated luminescence detection system, was used for all plate readings.

3.2.1.3.1 Fluorescent MUNANA-based assays

Fluorescent (FL) NA activity and inhibition assays were performed using the in-house MUNANA-based protocol developed by Public Health England (PHE)(Colindale, London, UK) (SOP NO. V-6815), and based on the method of Potier *et al.* [4]. The protocol is available to health professionals and to the general public at https://isirv.org/site/images/stories/avg_documents/Methodology/munana_ic50_sop_for_external.pdf. Black 96-well flat bottom plates were used with the 12-well side oriented vertically (rows 1 to 12, top to bottom) and the 8-well side oriented horizontally (columns A to H, right to left).

3.2.1.3.1.1 MUNANA neuraminidase activity assay

MES assay buffer (pH 6.5) containing 32.5mM MES (Sigma Aldrich, Schnelldorf, Germany) and 4mM $CaCl_2$ (MERCK, Darmstadt, Germany), was added to all 96 plate wells, adding 20µl per well. Duplicate two-fold virus dilutions were performed by adding 20µl of each virus isolate to the first row (row 1) of a pair of columns (from right to left, adding the first virus to wells A1 and B1, the second virus to wells C1 and D1 and so on until row 1 was filled; up to 4 different virus tested per plate) and gently mixing by pipetting up and down several times (½ starting dilution). The viruses were then serial diluted down the plate by transferring 20µl from row 1 to row 2 and so on, stopping at row 11 (½ to $\frac{1}{2048}$ dilution). The 20µl from row 11 were discarded and row 12 contained only MES buffer, being used as a blank control. A 100µM MUNANA substrate solution was prepared (5ml solution per plate), and 30µl of the fresh solution were added to all wells. To ensure that the components of each well were well mixed, the plate was carefully agitated by gentle tapping and centrifuged at 800rpm during 30 seconds (sec). An incubation period at 37ºC

for 60min with shaking and in the dark followed this quick centrifugation step. The reaction ended with the addition of 150µl of a stop solution (pH 10.7) containing 0.1M glycine (MERCK) and 25% ethanol (Carlo Erba Reagents, Milan, Italy) to all wells. The plate was then placed in the fluorometer and read within 20min of adding stop solution, using an excitation wavelength of 355nm and an emission wavelength of 460nm.
Raw activity data was analysed statistically (see section 3.2.2.1) to determine, for each virus isolate, the dilution in which enzyme activity yielded 35000 relative fluorescence units (RFUs). This standard NA activity was based on previous experiences that indicated its position within the linear range of the NA activity curve of most viruses. It usually implies a virus titre equal or higher than 16 haemagglutination units. Assay validation criteria included (1) the shape of the curve when plotting statistically treated RFUs against virus dilution (sigmoid); (2) replicate correlation (curve match and virus dilutions not differing more than one dilution factor), and; (3) control performance (values within the validation limits; detailed in section 3.2.2.1.2).

3.2.1.3.1.2 MUNANA neuraminidase inhibition assay

NA inhibition assay was performed on the same day or no more than 24 hours apart of the NA activity assay. Ideally, each virus isolate was assayed on two separate 96-well plates, to assess the susceptibility to OS and ZA at the same time. Duplicate dilutions of each virus were performed in MES assay buffer according to the dilution factor determined in previous activity assay (minimum total volume of 200µl per plate). All viruses that showed insufficient NA activity for inhibition testing (RFU of ½ dilution <35000) were not included and were submitted to further cell passage for increasing virus titre. Each diluted virus (up to 4 different virus per plate) was added to wells 1 to 11 of a pair of columns, adding 10µl per well (from right to left, the first virus was added to A+B wells 1-11, the second virus to C+D wells 1-11 and so on; Figure 3.1). An equal volume of MES buffer was added to all columns (A–H) of row 12. Four-fold dilutions of each antiviral (OS and ZA) were then prepared, beginning at 20000nM (Table 3.4). Each antiviral dilution was added to a full row of the plate, adding 10µl per well (from top to bottom, 20000nM dilution was added to row 1 of A-H columns, 5000nM dilution to row 2 of A-H columns and so on; Figure 3.1). To ensure that virus and antiviral were well mixed, the plate was carefully agitated by gentle tapping and centrifuged at 800rpm during

30sec. The plate was then incubated at 37ºC for 60min with shaking. All following steps, including preparation and addition of a fresh 100µM MUNANA solution, mixing, incubation, addition of stop solution and plate reading, were performed as in NA activity assay (see section 3.2.1.3.1.1).

Table 3.4 Antiviral dilution preparation in MUNANA neuraminidase inhibition assay.
The volumes indicated are for one working plate.

Step	Dilution series	Antiviral concentration (nM)	'In Assay' concentration (nM)
1	40µl of 100µM antiviral working solution + 160µl MES buffer	20000	4000
2	50µl of step 1 + 150 MES buffer	5000	1000
3	50µl of step 2 + 150 MES buffer	1250	250
4	50µl of step 3 + 150 MES buffer	312.5	62.5
5	50µl of step 4 + 150 MES buffer	78.13	15.63
6	50µl of step 5 + 150 MES buffer	19.53	3.91
7	50µl of step 6 + 150 MES buffer	4.88	0.98
8	50µl of step 7 + 150 MES buffer	1.22	0.24
9	50µl of step 8 + 150 MES buffer	0.31	0.061
10	50µl of step 9 + 150 MES buffer	0.076	0.015
11	MES buffer only	Virus/Substrate control	0
12	MES buffer only	Substrate/Buffer control (blank)	0

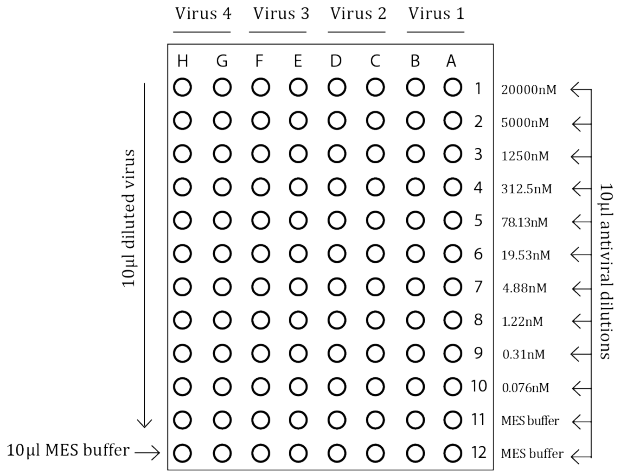

Figure 3.1 Plate layout for adding the viruses and antiviral dilutions in MUNANA neuraminidase inhibition assay.

Raw inhibition data was analysed statistically (see section 3.2.2.1) to determine, for each virus isolate and NAI, the concentration of antiviral required to inhibit the standardised

amount of virus NA activity by 50% (IC_{50}). Assay validation criteria included (1) the shape of the curve when plotting statistically treated RFUs against antiviral concentration (sigmoid); (2) replicate correlation (curve match and IC_{50} difference <30%); (3) NA activity in virus control wells (~35000RFUs); and, (4) control performance (IC_{50} values within the validation limits; detailed in section 3.2.2.1.2). IC_{50} values were then analysed statistically (detailed in section 3.2.2.3) to assess virus susceptibility phenotype to OS and ZA.

3.2.1.3.2 Chemiluminescent NA-Star® kit assays

Chemiluminescent (CL) methodology was used for confirmation of fluorescent-based susceptibility results, particularly of IC_{50} outlier values and whenever it was observed a significant difference between the results of inter-assay virus replicates.

NA activity and inhibition assays were carried out using the NA-Star® influenza NAI resistance detection kit (Applied Biosystems), following the manufacturer's instructions. The only exception were the antiviral dilutions that were prepared differently to match those use in fluorescent MUNANA assay (Table 3.5), and the final volume of NA-Star ® substrate working solution that was reduced to 2.5ml per plate. A quick centrifugation step (800rpm during 30sec) was also added to both activity and inhibition protocols after adding antiviral and/or substrate solution, to ensure that well contents were well mixed.

Table 3.5 Antiviral dilution preparation in NA-Star® kit neuraminidase inhibition assay.
The volumes indicated are for one working plate.

Step	Dilution series	Antiviral concentration (nM)	'In Assay' concentration (nM)
1	38.4µl of 100µM antiviral working solution + 361.6µl NA-Star® buffer	9600	4000
2	100µl of step 1 + 300µl NA-Star® buffer	2400	1000
3	100µl of step 2 + 300µl NA-Star® buffer	600	250
4	100µl of step 3 + 300µl NA-Star® buffer	150	62.5
5	100µl of step 4 + 300µl NA-Star® buffer	37.5	15.63
6	100µl of step 5 + 300µl NA-Star® buffer	9.38	3.91
7	100µl of step 6 + 300µl NA-Star® buffer	2.34	0.98
8	100µl of step 7 + 300µl NA-Star® buffer	0.59	0.24
9	100µl of step 8 + 300µl NA-Star® buffer	0.15	0.061
10	100µl of step 9 + 300µl NA-Star® buffer	0.037	0.015
11	NA-Star® buffer only	Virus/Substrate control	0
12	NA-Star® buffer only	Substrate/Buffer control (blank)	0

NA-Star® accelerator was quickly added at the end using a multi-channel pipette (luminescence reader not equipped with on-board injectors).

Raw activity assay data (obtained in relative light units (RLUs)) was analysed statistically (see section 3.2.2.1) to determine, for each virus isolate, the dilution that yielded a signal to noise ratio (S/N) of 40. This cut off was pre-established by the manufacturer (base-experience recommendation), usually implying a virus titre equal or higher than 16 haemagglutination units. Activity assays were only performed in an initial phase, until determining an optimal virus dilution for each influenza type or subtype. According to the manufacturer, similar and accurate IC_{50} values are achieved over a wide range of virus dilutions (100-fold difference in virus concentration). The $\frac{1}{20}$ dilution revealed to be optimal for former seasonal A(H1N1) (herein designated as seasonal A(H1N1)), A(H3N2), and influenza B viruses. Virus isolates with insufficient NA activity (S/N <40 in activity assay or in virus control wells in inhibition assay) were submitted to further cell passage for increasing virus titre. Raw data from inhibition assay (RLUs) was analysed statistically (see section 3.2.2.1) to determine the IC_{50} values, which were further analysed (see section 3.2.2.3) to assess virus susceptibility phenotype to OS and/or ZA.
Chemiluminescent assays were validated using the same criteria as in fluorescent MUNANA-based assays (S/N RLUs instead of treated RFUs in curve shape criterion; S/N~40 in virus control wells criterion).

3.2.1.4 Genotypic Molecular-Based Methods

3.2.1.4.1 Viral RNA extraction

Viral RNA was extracted from cell-culture isolates or clinical specimens using the QIAamp® Viral RNA Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. Purified RNA was recovered into 60µl nuclease-free water (Promega, San Luis Obispo, CA, USA) and used straightway or stored at -20ºC until further use.

3.2.1.4.2 Sanger sequencing

3.2.1.4.2.1 Reverse transcription - polymerase chain reaction amplification

Neuraminidase gene of former seasonal A(H1N1), A(H3N2) and influenza B viruses

A two-step reverse transcription - polymerase chain reaction (RT-PCR) protocol was used to amplify the complete coding region of the NA gene of seasonal A(H1N1), A(H3N2) and influenza B viruses. RT reaction was performed according to Ellis *et al.* [5]. Briefly, 22.2µl RNA were added to 17.8µl of a master mix containing, at a final concentration, 1x PCR buffer minus Mg (Invitrogen, Carlsbad, CA, USA), 7.5mM $MgCl_2$ (Invitrogen), 1.5mM of each deoxynucleoside triphosphate (dNTP; Roche, Barcelona, Spain), 0.4µM random hexamer primer (GE Healthcare, Little Chalfont Bucks, UK), 16U recombinant RNasin® ribonuclease inhibitor (Promega), and 200U Moloney murine leukemia virus reverse transcriptase (M-MLV RT; Invitrogen). Reaction conditions included an initial incubation step at room temperature for 10min (random primer extension), followed by two additional incubation steps, the first at 37°C for 45min (RT) and the second at 100°C for 5 min (denaturation), and a final cooling step at 8ºC. PCR amplification was performed with three primer pairs, specific of each influenza virus type or subtype (detailed in Table S3.1, Supplementary data). Each PCR reaction mixture included 10µl complementary DNA (cDNA) and 40µl of a master mix containing, at a final concentration, 1x PCR buffer minus Mg, 1mM (N1, NA B) or 1.25mM (N2) $MgCl_2$, 0.25µM (N1, NA B) or 0.1µM (N2) of each primer (Invitrogen), and 1.5U Taq DNA polymerase (Invitrogen). Thermal cycling conditions were performed as follows: 94ºC for 2min (initial denaturation); 35 cycles of 94ºC for 1min (denaturation), 50ºC (N1, N2) or 52ºC (NA B) for 1min (annealing), and 72ºC for 2min (extension); 72ºC for 5min (final extension); and cooling at 8ºC. Resulting amplicons were approximately 490 to 700 base pairs (bp) (Table S3.1, Supplementary data).

HA1 domain of hemagglutinin gene of former seasonal A(H1N1), A(H3N2) and influenza B viruses

The HA1 domain of seasonal H1, H3 and influenza B HA genes was amplified using a two-step RT-PCR protocol. RT reaction was performed as described above for full-length NA amplification. PCR primers were designed by PHE (Colindale, London, UK), including a single pair for either seasonal H1 or influenza B HA type/subtype and two different pairs for H3 HA subtype (see Table S3.2, Supplementary data for detail). PCR reaction mixture

was prepared by adding 10μl cDNA to 40μl of a master mix containing, at a final concentration, 1x PCR buffer minus Mg, 1.5mM $MgCl_2$, 0.2mM of each dNTP, 0.5μM of each primer (Invitrogen), and 0.75U Taq DNA polymerase. Thermal cycling conditions included: 94ºC for 2min; 35 cycles of 94ºC for 1min, 50ºC or 57ºC (H3 primer pair G/CII) for 1min and 72ºC for 2min; 72ºC for 5min; and cooling at 8ºC. Amplicons were approximately 760 to 1180bp (Table S3.2, Supplementary data).

Former seasonal A(H1N1), A(H3N2) and influenza B virus genome

All genome segments of seasonal A(H1N1), A(H3N2) and influenza B viruses, with exception of NA segment (methodology described above), were amplified using an optimized version of the one-step RT-PCR protocol developed by PHE (Colindale, London, UK) and kindly provided by Dr Monica Galiano. The influenza type-specific primer set used for PCR amplification was designed by Hoffmann *et al.* [6,7] and later redesigned by Dr Rod Daniels (NIMR, Mill Hill, London, UK). Protocol optimization involved adjustments in the annealing temperature of the primers that resulted in the use of a 49ºC to 60ºC temperature gradient instead of the single 55ºC annealing temperature. It also involved testing different combinations of primers. Final annealing temperatures and primer pairs are detailed in Table S3.3, Supplementary data.

Superscript™ III RT and Platinum® Pfx DNA polymerase kits (Invitrogen) were used for RT-PCR amplification. Each reaction mixture included 10μl RNA and 40μl of a master mix containing, at a final concentration, 1.5x Buffer Pfx (Pfx kit), 1mM $MgSO_4$ (Pfx kit), 0.4mM of each dNTP (Invitrogen), 0.2μM of each primer (Invitrogen), 20U recombinant RNasin® ribonuclease inhibitor, 200U Superscript™ III RT and 1.25U Platinum® Pfx DNA polymerase (proof-reading). Optimized cycling conditions were performed as follows: 50ºC for 30min (RT); 94ºC for 10min (initial denaturation); 35 cycles of 94ºC for 30sec (denaturation), 49ºC-60ºC for 30sec (annealing), and 68ºC for 2min (extension); 68ºC for 10min (final extension); and cooling at 8ºC. Amplicons were approximately 554 to 1594bp (Table S3.3, Supplementary data).

When the amplified products were undetectable or detectable at low amounts by gel electrophoresis (detailed in section 3.2.1.4.2.2), it was performed a nested PCR, also developed by PHE. Reaction mixture and cycling conditions were very similar to those used for RT-PCR. Differences included the absence of RNasin and Superscript RT from the

master mix and of a RT step in thermal cycling conditions, and the use of 2µl DNA to 48µl master mix.

2009 A(H1N1) pandemic influenza virus genome

Influenza A(H1N1)pdm09 virus genome segments were amplified using the one-step RT-PCR protocol developed by the Centers for Disease Control and Prevention (CDC)(Atlanta, GA, USA) and recommended and made available by WHO [8]. The protocol was used with minor modifications on primer pairs that included the use of different primer combinations for NA segment and a reduction in the number of primer pairs used for all other segments except the M segment. Also, some primer sequences were changed, by replacing one or few nucleotides with a degenerated one, to guarantee an optimal match to circulating viruses. Final primer pairs and sequences are shown in Table S3.4, Supplementary data. All forward (fw) and reverse (rev) primers included the sequence of, respectively, M13-fw and M13-rev universal primers, allowing the use of a posterior M13 universal sequencing strategy.

One-step RT-PCR reaction was performed using the AccessQuick™ RT-PCR kit (Promega) per the manufacturer´s instructions. Briefly, 15µl reaction mixtures were prepared by adding 2µl RNA to 13µl of a mixture containing, at a final concentration, 1x AccessQuick™ master mix (Tfl DNA Polymerase, dNTPs, $MgSO_4$ and reaction buffer), 1.5U avian myeloblastosis virus RT (AMV RT), and 1µM of each primer (Invitrogen). Thermal cycling conditions included: 48ºC for 45min; 94ºC for 2min; 30 cycles of 94ºC for 20sec, 50ºC for 30sec, and 72ºC for 1min; 72ºC for 7min; and cooling at 8ºC. Amplicons were approximately 447 to 790bp (Table S3.4, Supplementary data).

3.2.1.4.2.2 Agarose gel electrophoresis

RT-PCR products were size fractioned and visualized on GelRed-stained agarose gels. Gels were prepared using 1.2% agarose (SeaKem® LE agarose, Lonza, Rockland, ME, USA) and 0.25x GelRed™ (Biotium, Hayward, CA, USA) in 1x Tris-Borate-EDTA (TBE; 0.09M Tris, 0.09M Boric acid and 2.5mM EDTA). DNA samples were mixed with the appropriate volume of DNA loading buffer before being loaded into wells, using bromophenol blue/xylene cyanol DNA loading dye (0.25% bromophenol blue, 0.25%

xylene cyanol, 15% ficoll and 120mM EDTA) or 5x Orange G loading dye (Sigma-Aldrich). Electrophoresis was carried out at 80-120 volts (V) during 45-60min in 1x TBE buffer. DNA was then visualised by ultraviolet (UV) transillumination to check size and quality. The size of DNA bands was estimated by comparison with linear DNA standards of known molecular weight (100bp DNA ladder, Invitrogen) that ran along with samples. The intensity of staining and thickness of DNA bands were analysed to estimate the volume of DNA sample to be used in sequencing reaction when the posterior RT-PCR product purification was performed using ExoSAP-IT™ reagent (photometric DNA quantitation not possible).

3.2.1.4.2.3 DNA purification and quantitation

RT-PCR products were purified using the QIAquick PCR Purification kit (QIAGEN) or ExoSAP-IT™ PCR cleanup reagent (USB, GE Healthcare), according to the manufacturer's instructions. DNA purified with QIAquick kit was recovered into 30µl nuclease-free water and quantified (in µg/ml) by ultraviolet-visible spectrophotometry using a BioPhotometer. Photometric measurements were performed on 60µl of a 1:60 DNA dilution in nuclease-free water (blank).

3.2.1.4.2.4 Automated DNA sequencing

Nucleotide sequences were obtained by primer-walking (seasonal A(H1N1), A(H3N2), and influenza B; see Tables S3.1 to S3.3, Supplementary data for primer details) or M13 universal (A(H1N1)pdm09) sequencing approach. Cycle sequencing was performed using the BigDye® Terminator Cycle Sequencing kit v1.1 (Applied Biosystems) per the manufacturer´s instructions. Each reaction mixture contained 1µl BigDye® ready reaction mix, 3.2pmol sequencing primer (Invitrogen), 10ng (photometric quantification) or 0.5-2µl (band analysis) purified DNA, and nuclease free-water up to a volume of 10µl. Thermal cycling conditions included: 96ºC for 4min; 25 cycles of 96ºC for 10sec, 50ºC for 5sec, and 60ºC for 4min; 60ºC for 8min; and cooling at 8ºC. DNA sequences were then obtained using an automatic sequencer ABI PRISM® 3130xL Genetic Analyser (Applied Biosystems) after sample purification using 96-well DyeEx96 spin plates (DyeEx96 Kit,

QIAGEN), both according the manufacturer's instructions. These two last procedures were performed at the Technology and Innovation Unit, INSA, IP (Lisbon, Portugal).

3.2.1.4.3 Pyrosequencing

Pyrosequencing technology was used to (1) detect the molecular markers of resistance to M2 protein inhibitors (L26F, V27A, A30T/V, S31N/D and G34E; M gene, M2 protein-coding region); and (2) quantify NA H275Y and NA D197N amino acid variants in, respectively, A(H1N1)pdm09 and influenza B virus quasispecies (NA gene).
Pyrosequencing reaction was performed after RT-PCR amplification and sample preparation. All procedures were carried out at the PHE (Colindale, London, UK), using in-house protocols. Influenza A M2 amplification and pyrosequencing protocols were the original pyrosequencer manufacturer's protocols, described in Bright *et al.* [9], with minor modifications. Both RT-PCR reaction mixture and cycling conditions were adjusted for A(H1N1)pdm09 NA H275Y (protocol available at https://isirv.org/site/index.php/methodology/pyrosequencing) and influenza B NA D197N pyrosequencing, using the PyroMark® Assay Design SW 2.0 tool (QIAGEN) for primer design. PyroMark® Q96 Vacuum workstation and ID pyrosequencer (QIAGEN) were used for, respectively, sample preparation and pyrosequencing reactions. Both equipments were used per the manufacturer's instructions.

3.2.1.4.3.1 One-step reverse transcription - polymerase chain reaction

RT-PCR was performed using the QIAGEN® OneStep RT-PCR kit according to the manufacturer´s instructions and a single primer pair, in which one of the primers was biotin-labelled at its 5' end (see Table S.3.5, Supplementary data for detail). Each reaction mixture was prepared by adding 5µl (M2) or 2.5µl (NA) RNA to a master mix containing 10µl 5x QIAGEN OneStep RT-PCR buffer, 2µl dNTP mix (QIAGEN kit), 3µl of each 10µM primer (Metabion, Martinsried, Germany), 2µl QIAGEN OneStep RT-PCR enzyme mix, and RNase-free water (QIAGEN kit) up to a volume of 45µl (M2) or 47.5µl (NA). Thermal cycling conditions were performed as follows: 50ºC for 30min; 95ºC for 15min; 35 (M2) or 40 (NA) cycles of 94ºC for 1min (M2)/30sec (NA), 55ºC for 1min (M2) or 62ºC for 30

sec (NA), and 72ºC for 1min; 72ºC for 10min; and cooling at 8ºC. Biotinylated amplicons were 264bp for M2, covering the region extending between nucleotides 764 and 1027, and 242bp for NA, covering the region extending between nucleotides 800 to 1041 (A(H1N1)pdm09) or 483 to 724 (influenza B).

3.2.1.4.3.2 Sample preparation

Biotin-labelled RT-PCR products were cleaned and denatured into single-stranded DNA. A master mix containing, per sample, 3µl streptavidin Sepharose™ high performance beads (GE Healthcare, Little Chalfont Bucks, UK), 17µl PyroMark binding buffer (QIAGEN) and 40µl nuclease-free water, was prepared and distributed across a 96 deep-well PCR plate (60µl/well). RT-PCR products were added into each well of the plate, adding 20µl of product. The plate was incubated at room temperature for 10min while shaking at 1400rpm, for immobilisation of the amplified products to the streptavidin coated sepharose beads (biotin-streptavidin bond). Single-stranded DNA products were then obtained using the PyroMark® Q96 Vacuum workstation. DNA-coated beads were collected using the 96-pin vacuum tool and were put through a series of steps that included (1) washing with 70% ethanol; (2) denaturing of the double-stranded DNA in 0.2M NAOH to leave only the labelled single-stranded DNA coated to the beads; and (3) washing with 10mM Tris Acetate pH 7.6 buffer to remove any unbound DNA. Single stranded DNA-coated beads were then released into a PyroMark® Q96 well plate low (QIAGEN) containing 0.44µM sequencing primer (Metabion; detailed in Table S3.5, Supplementary data) in 45µl PyroMark® annealing buffer (QIAGEN), by aligning the vacuum tool with the plate and turning off the pump. PyroMark plate was incubated at 80ºC for 2min and then allowed to cool to room temperature for approximately 10min before being placed into the pyrosequencing machine.

3.2.1.4.3.3 Pyrosequencing reaction

Pyrosequencing reaction was performed on a PyroMark Q96 ID instrument (QIAGEN) using the PyroMark® Gold Q96 Reagent Kit (enzyme mixture, substrate mixture, dATPαS, dGTP, dCTP, dTTP; QIAGEN) according to the manufacturer´s instructions. M2

pyrosequencing target region was 44bp, extending between nucleotides 784 and 827 of the M gene of influenza A viruses (seasonal A(H1N1), A(H3N2); encoding amino acids 25 to 38 of M2 protein). NA pyrosequencing assays targeted shorter regions that comprised a 16bp region for A(H1N1)pmd09 NA H275Y assay (nucleotides 812 to 827; amino acids 271 to 275); and a 20bp region for influenza B NA D197N assay (nucleotides 581 to 600; amino acids 195 to 200). Pyrosequencing runs were performed using single nucleotide polymorphism (SNP) analysis, with the machine generating the nucleotide dispensation order automatically, based on the expected sequence. Sequencing reaction occurred at 28ºC by stepwise elongation of the primer upon cyclic dispensation of the four different dNTPs in the pre-specified order. A limit of 10% was considered for SNP detection.

3.2.2 Statistical Analysis

3.2.2.1 Phenotypic Neuraminidase Inhibition Assays

3.2.2.1.1 Optimal virus dilution and IC_{50} determination

Raw phenotypic NA activity assay data was analysed statistically to determine the optimal virus dilution to be used in following NA inhibition assay (dilution yielding 35000 RFUs (FL assay) or S/N=40 (CL assay)), using Microsoft Office Excel 2007 (Microsoft Office Professional Edition 2007). The mean value for the blank buffer control wells (row 12) was calculated and taken from each data point (FL assay) or used to divide each data point (CL assay). Data was then plotted as RFUs (FL assay) or RLUs (CL assay) against virus dilution, yielding a sigmoid dose-response curve, and the optimal virus dilution was determined by point-to-point curve fitting, using the log10 of the virus dilution values. Each virus replicate was plotted independently and the optimal virus dilution was determined for each replicate separately, taking the mean value as the final value. In FL assay, this mean value was further divided by two as only half of the virus volume used in activity assay (20µl) is used in the following inhibition assay (10µl).
A similar statistical treatment was applied to raw NA inhibition assay data, plotting RFU or RLU data against NAI drug concentration and using the log10 of the drug concentration values for point-to-point curve fitting analysis. The NA activity given by

50% of the virus control value (row 11) was determined and used as cut-off for IC_{50} determination.

3.2.2.1.2 Validation limits for virus control performance

Validation limits for virus control performance in both NA activity and inhibition assays were established at 3 standard deviations (SD) above and below the median, using the log10 of optimal virus dilution or IC_{50} value in all calculations. The limits were established with a minimum of 10 log10 values from 10 independent assays.

3.2.2.1.3 IC_{50} Data Analysis

Log10 IC_{50} values were calculated and used for determining upper and lower cut-offs by scaled median absolute deviation (SMAD) robust method, according to the recommendations of the Analytical Methods Committee of the Royal Society of Chemistry [10]. Each cut-off comprised two levels: a mild level, established at 1.65 SD above (upper) or below (lower) the median; and an extreme level, established at 3 SD above (upper) or below (lower) the median. Influenza viruses exhibiting an IC_{50} value above and below upper and lower mild/extreme cut-offs, respectively, were considered as outlier. All viruses classified as outlier were retested twice and the mean IC_{50} was taken as the final value. Once identified, outlier IC_{50} values were removed from data to calculate the IC_{50} baseline median. IC_{50} cut-offs and baseline median were determined for each influenza virus type or subtype and for each influenza season or time period (2009 pandemic period), using a minimum of 15 IC_{50} values from different virus isolates. When less than 15 isolates were assayed, IC_{50} values were analysed against the cut-offs and median baseline estimated for the influenza type or subtype-matched viruses from the previous or following influenza season. Overall IC_{50} baseline median was determined for each influenza virus type or subtype and influenza B lineage. IC_{50} values were further divided by the baseline median to determine the fold difference and classify the viruses according to the new standardised definitions for reporting NAI susceptibility data - normal inhibition (NI) (fold-change increase <10 (influenza A) or <5 (influenza B)); reduced inhibition (RI) (fold-change increase 10-100 (influenza A) or 5-50 (influenza B)); and

highly reduced inhibition (HRI) (fold-change increase >100 (influenza A) or >50 (influenza B)) [11].

The standard error (SE) of IC_{50} baseline (or outlier) median estimates was calculated manually by dividing the SD of the IC_{50} values, previously determined through SMAD statistical method, by the square root of the sample size. The existence of statistically significant differences among the IC_{50} baseline (or outlier) median of circulating influenza viruses over time, was initially assessed by a Kruskal-Wallis H test and then, if confirmed, by post hoc pairwise comparisons using Dunn's (1964) procedure with a Bonferroni adjustment. When at least one of the median estimates was based on less than 5 values ($n<5$), pairwise comparisons were exceptionally performed using multiple Mann-Whitney U tests to get the exact p-value. The Mann-Whitney U test was also used when only two median estimates were available for comparison. The same statistical approach was used to compare the IC_{50} baseline median of influenza viruses from different types or subtypes/lineages, either within each influenza season/time period or during the overall time period. OS and ZA IC_{50} baseline median estimates were compared through a Wilcoxon Signed-Ranked test, excluding all non-paired IC_{50} values from the analysis. Asymptotic (standard) and exact p-values were considered statistically significant at ≤0.05. All statistical analyses were performed in SPSS v22.0 software (IBM, Armonk, New York, USA; http://www.ibm.com/software/analytics/spss), using log10-transformed non-outlier IC_{50} values.

3.2.2.2 Frequency of Influenza Antiviral Drug Prescription

The frequency of influenza antiviral drug prescription in ILI patients presenting to healthcare systems covered by the National Influenza Surveillance Programme was estimated for the 2005/2006 to 2008/2009 influenza seasons, using the information available in ILI notification forms. Estimated frequencies were then compared in SPSS v17.0 software using a Person Chi-Square test and considering a p-value ≤0.05 for statistical significance.

3.2.3 Bioinformatics

3.2.3.1 Sanger Sequencing

Sequence trace files generated by automated DNA sequencing were viewed and checked for size and quality using Applied Biosystems Sequence Scanner software v1.0 (https://products.appliedbiosystems.com). Once checked, trace files were assembled and edited using the SeqMan program from Lasergene software package v7.0.0 (DNASTAR, Madison, WI, USA; http://www.dnastar.com), to obtain the consensus DNA protein-coding sequence.

3.2.3.2 Alignment of Sequences and Mutational Analysis

Sequence alignments were performed by Clustal W method in MEGA5 software v5.2.2 (http://www.megasoftware.net), including sequences from influenza reference viruses retrieved from GISAID EpiFlu™ or NCBI Influenza Virus Resource Database. Reference viruses included the vaccine viruses for the Northern Hemisphere 2004/2005 to 2012/2013 influenza seasons and other reference viruses from the same period, defined in the WHO reports for the annual consultations on the composition of influenza vaccines (available at https://www.crick.ac.uk/research/worldwide-influenza-centre/annual-and-interim-reports/). Sequences from earlier reference viruses (3 to 4 sequences) were also included to root the phylogenetic trees (outgroup) and provide directionality to the evolutionary histories. Detailed information on the reference sequences used, including accession number, is presented in the supplementary data of chapter 4 (Table S4.2) and chapter 5 (Table S5.2). Once aligned, sequences were manually checked and edited to remove any non-coding region, and were further translated into their amino acid sequence. Amino acid substitutions were then identified against the corresponding consensus sequence. All these latter procedures were performed in MEGA5 software. Whenever possible, amino acid substitutions were mapped onto the three-dimensional structure of the protein, using PyMOL software educational version 1.3. Protein structure files were retrieved from the RCSB Protein Data Bank (PDB) (http://www.rcsb.org/pdb).

3.2.3.3 Phylogenetic Analysis

Phylogenetic trees of the NA and HA genes of each influenza virus type or subtype were constructed by maximum-likelihood (ML) method, using the PhyML3.0 platform available in SeaView software package v.4.4.0 (http://doua.prabi.fr/software/seaview), after determining the best-fit model of nucleotide substitution. This model was determined in jModelTest program v.2.1.2 (https://code.google.com/p/jmodeltest2/), according to Akaike's information criterion (AIC), using a 95% confidence interval and likelihood scores estimated with 11 substitution schemes (88 candidate models). Model selection was performed for all different sets of influenza virus type or subtype aligned sequences. Based on the results obtained, it was then selected the best-fit agreed model for each gene. Branch support values were estimated by an approximate likelihood-ratio test (aLRT) based on a Shimodaira-Hasegawa-like procedure (aLRT (SH-like)). Both subtree-pruning-and-regrafting (SPR) and nearest-neighbour-interchange (NNI) rearrangement operations were selected to optimise tree topology, using 10 random starting trees. Once constructed, ML trees were visualized and edited using FigTree program v1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/) and further annotated using Adobe Illustrator CS5 (http://www.adobe.com/products/illustrator.html).

3.2.3.4 Selective Pressure Analysis

The worldwide NA gene sequences collected for each time period (detailed above in section 3.1.6), were subjected to a 5-step sequence treatment that included: (1) identifying the sequences shared by GISAID EpiFlu™ and NCBI Influenza Virus Resource databases and the sequences repeated within each one; (2) removing the shared sequences from one of the source files using MEGA5 software; (3) merging the three sequence source files (GISAID, NCBI, Portugal) available for each time period and influenza virus subtype or lineage, aligning the sequences by Clustal W method and removing all sequences containing gaps or with a not complete coding region in MEGA5; (4) removing redundant sequences (100% threshold) and sequences containing degenerate or untranslatable nucleotides, using Jalview program v2.8 (http://www.jalview.org); and (5) removing the repeated sequences initially identified if still present in the final sub-dataset, using MEGA5. An additional step was performed between steps 2 and 3 for the influenza B NA sequences retrieved from NCBI Influenza Virus Resource database, as no lineage information was available for most sequences.

The sequences were aligned with B/Victoria and B/Yamagata-lineage reference sequences by Clustal W method in MEGA5. Reference sequences were selected according to the WHO annual reports (see section 3.2.3.2) and retrieved from GISAID EpiFlu™ database. A ML phylogenetic tree was then constructed as described in previous section 3.2.3.3 and a B lineage was assigned to each virus sequence, according to its position on the tree. Total sequence datasets were constructed by merging treated temporal sub-datasets. A sequence similarity threshold of 99.99% was further applied to A(H3N2) and A(H1N1)pdm09 datasets to reduce the number of total sequences to below 4000, which was the limit of the program later used for SP analysis. Sequence reduction was performed using the CD-HIT-EST program from CD-HIT software package, available on the web server CD-HIT Suite (http://weizhong-lab.ucsd.edu/cdhit_suite).

Sequence diversity (number of base substitutions per site from a mean of all sequence pairs) was estimated by Maximum Composite Likelihood method in MEGA5, using the standard gamma distribution (shape parameter = 1.327) of rate variation among sites. A total of 500 bootstrap replications were used to estimate the associated standard error. The presence of potential recombination breakpoints was further analysed in total sequence datasets by genetic algorithm recombination detection method, using HyPhy software v2.2 (http://hyphy.org; SingleBreakpointRecomb.bf batch file). Since no single phylogenetic tree can accurately describe the evolutionary relationships of recombinant sequences, it was important to ruled out or accounted for recombination as its presence could mislead inferences of selection [12].

ML phylogenetic trees were inferred as described in section 3.2.3.3, selecting the best-fit model of nucleotide substitution based on seasonal A(H1N1) total sequence dataset. Phylogenetic trees of sequence alignments with more than 3000 sequences were exceptionally inferred in RAxML version 8, using standard nonparametric bootstrap (500 replicates) for estimating branch support values.

All SP analyses were carried out in HyPhy software, after determining the time-reversible model that, composed with Muse-Gaut 94 model, resulted in the best-fit codon model for influenza NA. Model selection was performed by analysing the seasonal A(H1N1) total sequence dataset through CodonModelCompare.bf procedure, using AIC. SP analyses included: (1) estimating the global ratio between non-synonymous (dN) and synonymous (dS) substitution rates (dN/dS ratio) with 95% confidence intervals, using the standard AnalyzeCodonData.bf procedure; (2) estimating the site-specific dN/dS ratios and identifying the sites under positive selection by both single-likelihood ancestor counting

(SLAC) and two rate fixed effects likelihood (FEL) methods, available in QuickSelectionDetection.bf batch file; and (3) identifying the codons under different SP among the different time periods studied (differential selection analysis), using the CompareSelectivePressure.bf procedure. A significance level of ≤0.05 was set for all analyses. Positively selected sites were further mapped onto the three-dimensional structure of the influenza NA subtype or lineage in which were detected, using the methodology previously described for amino acid substitutions (see section 3.2.3.1).

3.3 REFERENCES

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SUPPLEMENTARY DATA

Table S3.1 Primer set used for full-length amplification and sequencing of the neuraminidase gene of former seasonal A(H1N1), A(H3N2) and influenza B viruses.

Influenza virus type/subtype	Segment	PRIMER			PROCEDURE		EXPECTED SIZE (bp)	
		Designation	Direction	Sequence (5´- 3´)	Amplification	Sequencing	Amplicon	Sequence
A(H3N2)	N2 - 1	NA2-1	Fw	AGCAAAAGCAGGAGTAAAGAT	x	x	515	515
		NA2-515	Rev	GAACACCCAAYTCATTCATCAATA	x	x		515
	N2 - 2	NA2-386	Fw	TCATGCGATCCTGACAART	x	x	579	579
		NA2-964	Rev	ACTGGAAACAATGCTATAATCCTT	x	x		579
	N2-3	NA2-770	Fw	GATACTAAAATACTATTCATTGAG	x	x	698	698
		NA2-1467	Rev	AGTAGAAACAAGGAGTTTTT	x			-
		NA2-1001	Fw	AAAAACGACAGCTCCAG		x	-	467
A(H1N1)	N1-1	NA1-1	Fw	AGCAAAAGCAGGAGTTTA	x	x	659	659
		NA1-659	Rev	AGTTATTATKCCGTTGTATTTTAG	x			-
		NA1-452	Rev	ATGTTTGTCATTTAATAGAGCA		x	-	452
		NA1-351	Fw	AAAGGAGATGTTTTTGTCATA		x	-	309
	N1-2	NA1-535	Fw	CAAAGTTTGAATCAGTTGC	x	x	494	494
		NA1-1028	Rev	ATTACAGCTGCCCTYTCCATCTTT	x	x		494
	N1-3	NA1-888	Fw	TGTGTATGCAGGGACAACT	x	x	576	576
		NA1-1463	Rev	AGTAGAAACAAGGAGTTTTT	x			-
		NA1-1104	Fw	ACTAAAAGTAACAGACTTAGAAAG		x	-	360
B	B-1	1B	Fw	CCAAAATGAACAATGCTACC	x	x	653	653
		653B	Rev	TATGTGTCAGTRTATGCTTCTCCA	x	x		653
		359B	Rev	CTTATTATCAAGGGAGCTGAGTTT		x	-	359
	B-2	495B	Fw	RGGCAAAATMCCAACAGTAG	x	x	545	545
		1039B	Rev	TGTYCCCATTAGATTCRCAAG	x	x		545
	B-3	857B	Fw	CYAGCAATAAAACCATAGAATGTG	x	x	607	607
		1463B	Rev	TCAAACAAAATAGGAACAAAG	x			-
		1107B	Fw	CTCTCGAACRATGTCTAAAACYAA		x	-	357

bp: base pairs; Fw: Forward; Rev: Reverse

The x indicates in which procedure the primer was used.

Table S3.2 Primer set used for amplification and sequencing of the HA1 domain of the hemagglutinin gene of former seasonal A(H1N1), A(H3N2) and influenza B viruses.

Influenza virus type/subtype	Segment	PRIMER			PROCEDURE		EXPECTED SIZE (bp)	
		Designation	Direction	Sequence (5´- 3´)	Amplification	Sequencing	Amplicon	Sequence
A(H3N2)	A	AH3G	Fw	AAGCAGGGGATAATTCTATT	x	x	932	≈700 [a]
		AH3CII	Rev	GCTTCCATTTGGAGTGATGC	x	x		≈700 [a]
	B	AH3B	Fw	AGCAAAGCTTACAGCAACTG	x	x	767	≈700 [a]
		AH3I	Rev	TCCCTCCCAACCATTTTCCTA	x	x		≈700 [a]
A(H1N1)	-	AH1G	Fw	AAGCAGGGGAAAATAAAAAC	x	x	1121	≈700 [a]
		AH1H	Rev	CCATCCATCTATCATTCCAG	x	x		≈700 [a]
		AH1M	Rev	TGATGCGTTTGAGGTGATGAT		x	-	≈700 [a]
B	-	BHAE	Fw	GAAGCAGAGCATTTTCTAAT	x	x	1168	≈700 [a]
		BHAG	Rev	ATCATTCCTTCCCATCCTCC	x	x		≈700 [a]
		BHAB-5	Fw	GCACGACAGAACAAAAAT		x	-	≈700 [a]

bp: base pairs; Fw: Forward; Rev: Reverse

[a] Maximum length for sequence readings with good quality

The x indicates in which procedure the primer was used.

Table S3.3 Primer set used for amplification and sequencing of all genome segments except NA of former seasonal A(H1N1), A(H3N2) and influenza B viruses, with indication of the corresponding annealing temperatures.

	Segment			PRIMER			PROCEDURE			EXPECTED SIZE (bp)	
			Designation	DIR	Sequence (5´- 3´)	AMP	Ta (ºC)	SEQ	Amplicon	Sequence
INFLUENZA A	PB2	PB2-1(A)	Bm-PB2-1	Fw	TATTGGTCTCAGGGAGCRAAAGCAGGTC	x	58 (H3N2)		974	-
			HPB2R960	Rev	GCATATRTCCACAGCTTGTTCTTC	x	55 (H1N1)	x		≈700 [a]
			HPB2R421	Rev	GGCCAAAGGTTCCATGTTTTAACC		-	x	-	435
		PB2-1(B) A(H3N2) (amp) type A (seq)	HPB2F841	Fw	GTATCAGCAGATCCACTAGCATCT	x	49	x	619 (H3N2)	619
		PB2-1(B) A(H1N1)	HPB2F398	Fw	GGTTAAAACATGGAACCTTTGGCC	x			1062 (H1N1)	-
		PB2-1(B) type A	HPB2R1459	Rev	TCCTCTCATTGACATCTC	x				-
		PB2-2	PB2-1105F	Fw	TAYGARGARTTCACAATGGT	x	52 (H3N2)	x	1252	≈700 [a]
			Bm-PB2-2341R	Rev	ATATGGTCTCGTATTAGTAGAAACAAGGTCGTTT	x	55 (H1N1)			-
			HPB2F1441	Fw	GAGATGTCAATGAGAGGA		-	x	-	≈700 [a]
			HPB2F1785	Fw	GGCCATTAGAGGCCAATACAGTGG		-	x	-	557
	PB1	PB1-1	Bm-PB1-1	Fw	TATTGGTCTCAGGGAGCRAAAGCAGGCA	x	55 (H3N2)	x	1276	≈700 [a]
			PB1-1262R	Rev	TTRAACATGCCCATCATCAT	x	58 (H1N1)			-
			HPB1F370	Fw	CAAACAAGGGTGGACAAACT		-	x	-	≈700 [a]
			HPB1R389	Rev	AGTTTGTCCACCCTTGTTTG		-	x	-	403
		PB1-2	PB1-1123F	Fw	CARATACCNGCAGARATGCT	x	52 (H3N2)	x	1234	≈700 [a]
			Bm-PB1-2341R	Rev	ATATGGTCTCGTATTAGTAGAAACAAGGCATTT	x	55 (H1N1)			-
			HPB1F1711	Fw	AGAGGWGACACACAAATWCA		-	x	-	647
	PA	PA-1	Bm-PA-1	Fw	TATTGGTCTCAGGGAGCRAAAGCAGGTAC	x	55 (H3N2)	x	1512	≈700 [a]
			PA-1498R	Rev	TNGTYCTRCAYTTGCTTATCAT	x	52 (H1N1)			-
			HPAF597	Fw	TCGTCAGTCCGAAAGAGGCGAAGA		-	x	-	≈700 [a]
			HPAR620	Rev	TCTTCGCCTCTTTCGGACTGACGA		-	x	-	634
		PA-2(B)	HPAF960	Fw	AACATTCTTTGGATGGAAAGAACC	x	55 (H3N2)	x	1289	≈700 [a]
			Bm-PA-2233R	Rev	ATATGGTCTCGTATTAGTAGAAACAAGGTACTT	x	52 (H1N1)			-
			HPAF1435	Fw	AATGCATCCTGTGCAGCAATGGA		-	x	-	≈700 [a]
			HPAF1801	Fw	GAGAGCATGATTGAAGCYGAGTCC		-	x	-	448
	HA	HA-1 type A	HAFUc	Fw	TATTCGTCTCAGGGAGCAAAAGCAGGGG	x	55 (H3N2)		664 (H3N2)	-
		HA-1 A(H3N2)	H3HAR650	Rev	TTGGTCACTGTCCGTACTCGGGTG	x	52 (H1N1)	x	1101 (H1N1)	664
		HA-1 A(H1N1)	H1HAR1087	Rev	AAACCGGCAATGGCTCCAAA	x		x		≈700 [a]
		HA-1 A(H1N1)	H1HAR623	Rev	GATGAACACCCCATAGTACAAGGA		-	x	-	637
		HA-2 A(H3N2)	H3HAF567	Fw	CTGAACGTGACTATGCCAAACAAT	x	55 (H3N2)	x	1211 (H3N2)	≈700 [a]
		HA-2 A(H1N1)	H1HAF552	Fw	TACCCAAACCTGAGCAAGTCCTAT	x	52 (H1N1)	x	1239 (H1N1)	≈700 [a]
		HA-2 type A	HARUc	Rev	ATATCGTCTCGTATTAGTAGAAACAAGGGTGTTTT	x				-
		HA-2 A(H3N2)	H3A2F1	Fw	AGGCATATTCGGCGCAATCGCAGG		-	x	-	≈700 [a]
		HA-2 A(H1N1)	H1HA2F1	Fw	ACATCCCATCCATTCAATCCAGAG		-	x	-	≈700 [a]
	NP	-	NPFUc	Fw	TATTCGTCTCAGGGAGCAAAAGCAGGGTWRATAATC	x	58 (H3N2)	x	1594	≈700 [a]
			NPRUc	Rev	ATATCGTCTCGTATTAGTAGAAACAAGGGTAWTTTT	x	55 (H1N1)			-
			HNPR509	Rev	ACAAGAGCTCTTGTCCTCTGGTA		-	x	-	523
			NPUF525-48	Fw	CCCAGRATGTGYTCYYTRATGCA		-	x	-	≈700 [a]
			HNPF986	Fw	CCTAATCAGACCDAACGAGAATCC		-	x	-	595
	M	-	MFUc	Fw	TATTCGTCTCAGGGAGCAAAAGCAGGTAGA	x	58 (H3N2)		1056	-
			MRUc	Rev	ATATCGTCTCGTATTAGTAGAAACAAGGTAGTTTTT	x	60 (H1N1)	x		≈700 [a]
			HMR400	Rev	GGCAAGTGCACCAGCAGAATA		-	x	-	414
			HMF581	Fw	GCTAAGGCTATGGAGCAAATGGCT		-	x	-	462
	NS	-	NSFUc	Fw	TATTCGTCTCAGGGAGCAAAAGCAGGGTG	x	55 (H3N2)	x	919	≈700 [a]
			NSRUc	Rev	ATATCGTCTCGTATTAGTAGAAACAAGGGTGTTTTTT	x	58 (H1N1)			-
			HNSF481	Fw	GAAGAGGGAGCAATTGTTGGCGAA		-	x	-	425
			HNSR504	Rev	TTCGCCAACAATTGCTCCCTCTTC		-	x	-	518

(Table S3.3 cont.)

	Segment		PRIMER			PROCEDURE			EXPECTED SIZE (bp)	
			Designation	DIR	Sequence (5´- 3´)	AMP	Ta (ºC)	SEQ	Amplicon	Sequence
INFLUENZA B	PB2	PB2-1	BPB2F1U	Fw	TATTCGTCTCAGGGAGCAGAAGCGGAGCGTTTTCAAGATG	x	55	x	1247	≈700 [a]
			BPB2R1233	Rev	CCTAGTGTCTTGAGAAAATACCAT	x		x		≈700 [a]
			BPB2_177R	Rev	ATGTCACCCTTGGTYARAGC		-	x	-	191
		PB2-2(A)	BPB2F1102	Fw	ATACAGAARATTGGAATATGGCA	x	49		569	-
			BPB2_1670R	Rev	CTGAGCCTTCAGTGTTACCAAA	x		x		569
		PB2-2(B)	BPB2_1385F	Fw	ATCACCCAAAGCAAGTGARC	x	55	x	941	≈700 [a]
			BPB2REU	Rev	ATATCGTCTCGTATTAGTAGAAACACGAGCATTTTTCAC	x				-
			BPB2_1875F	Fw	TTGCCCTTTTGTTTCTCACC		-	x	-	451
	PB1	PB1-1	BPB1F1U	Fw	TATTCGTCTCAGGGAGCAGAAGCGGAGCCTTTAAGATG	x	58		1269	-
			BPB1R1255	Rev	CATATTAAACATTCCCATCATCAT	x				-
			BPB1_351F	Fw	TGGAGGCACTAATGGTCACA		-	x	-	≈700 [a]
			BPB1_854F	Fw	GAAGGCCAAACTGTCAAAYGC		-	x	-	402
			BPB1_687R	Rev	TAGTTTGCCCCTTTCAGCRT		-	x	-	701
		PB1-2	BPB1F1031	Fw	ACCAGAGACAGCCCAATTTGGTT	x	55		1272	-
			BPB1REU	Rev	ATATCGTCTCGTATTAGTAGAAACACGAGCCTTTTTTCA	x				-
			BPB1_1357F	Fw	TTGCTCTGTTTGTTAATGCAAAA		-	x	-	≈700 [a]
			BPB1_1881F	Fw	GGCGATTACTTCACCCTCAA		-	x	-	422
			PPB1_1669R	Rev	CTCCTCTGTGGCATTTGTAG		-	x	-	639
	PA	PA-1	BPAF1U	Fw	TATTCGTCTCAGGGAGCAGAAGCGGTGCGTTTGATTTG	x	55		1406	-
			BPAR1392	Rev	GTGTGRAAAAGYACATACTTCATCAT	x				-
			BPA_330F	Fw	CCAAAGTATCTGGCTGATTTG		-	x	-	≈700 [a]
			BPA_642R	Rev	GGAGAAACCAGCTGGAACAG		-	x	-	657
		PA-2(B)	BPA_803F	Fw	AAGACCAATAGGGCCTCACA	x	49		1472	-
			BPAREU	Rev	ATATCGTCTCGTATTAGTAGAAACACGTGCATTTTTRAT	x				-
			BPA_1308F	Fw	CCGTGGAGCATGTAGGGAGT		-	x	-	≈700 [a]
			BPA_1816F	Fw	TGACCAAAGCTTGTTTCAAGG		-	x	-	459
			BPA_1631R	Rev	TTTTCCCTCCCACTCACAAA		-	x	-	≈700 [a]
	HA	HA(A)	BHAF1U	Fw	TATTCGTCTCAGGGAGCAGAAGCAGAGCATTTTCTAATATC	x	49	x	1182	≈700 [a]
			BHA_1168R	Rev	ACTGCYACTCCATGTGCYC	x		x		≈700 [a]
			BHA_680R	Rev	CTCCATTRGCAGATGAGGTGAA		-	x	-	694
		HA(B)	BHA_868F	Fw	GTTGCCTCAAAAGGTGTGGT	x	52	x	1062	≈700 [a]
			BHAREU	Rev	ATATCGTCTCGTATTAGTAGTAACAAGAGCATTTTTCAAT	x		x		≈700 [a]
	NP	NP(A)	BNPF1U	Fw	TATTCGTCTCAGGGAGCAGAAGCASAGCATTTTCTYGTG	x	55		633	-
			BNP_619R	Rev	GCCTTTGATCTTTGGAAACAG	x		x		633
		NP(B)	BNP_308F	Fw	GGACTCAACGATGACATGGA	x	49	x	1326	≈700 [a]
			BNP_1633R	Rev	CTGTGTCCCTCCCAAAGAAG	x				-
			BNP_805F	Fw	GGCAGACAGAGGGCTATTGA		-	x	-	≈700 [a]
		NP(C)	BNP_1320F	Fw	TGATGTCCATCAAGCTCCAG	x	52	x	570	570
			BNPREU	Rev	ATATCGTCTCGTATTAGTAGAAACAACAGCATTTTTTACA	x				-
	M	M(A)	BMF1U	Fw	TATTCGTCTCAGGGAGCAGAAGCASGCACTTTCTTAARATG	x	49		578	-
			BM_564R	Rev	TCCTTTTCCCATTCCATTCA	x		x		578
		M(B)	BM_256F	Fw	CACAGAGCCCCTATCAGGAA	x	55	x	981	≈700 [a]
			BMREU	Rev	ATATCGTCTCGTATTAGTAGAAACAACGCACTTTTTCCAG	x				-
			BM_768F	Fw	TGCTCGAACCATTTCAGATTC		-	x	-	469
	NS	-	BNSF1U	Fw	TATTCGTCTCAGGGAGCAGAAGCAGAGSATTTGTTTAGTC	x	55		1157	-
			BNSREU	Rev	ATATCGTCTCGTATTAGTAGTAACAAGAGGATTTTTATT	x				-
			BNS_203F	Fw	TCACAACAAAAGTGAGCCTGA		-	x	-	≈700 [a]
			BNS_727F	Fw	CCATCGGATCCTCAACTCAC		-	x	-	417
			BNS_512R	Rev	CGTATATCCCTTTTTATTGTCAAACG		-	x	-	526

AMP: Amplification; bp: base pairs; DIR: Direction; Fw: Forward; Rev: Reverse; SEQ: sequencing; Ta: annealing temperature

[a] Maximum length for sequence readings with good quality

The x indicates in which procedure the primer was used.

Table S3.4 Primer set used for amplification of influenza A(H1N1)pdm09 virus genome segments.

Segment		FORWARD PRIMER		REVERSE PRIMER		Expected size amplicon (bp)
		Designation	Sequence (5´- 3´)	Designation	Sequence (5´- 3´)	
PB2	1	1	*TGTAAAACGACGGCCAGT*CTCGAGCAAAAGCAGGTCAA	575	*CAGGAAACAGCTATGACC*YAGCTGTGAYTCYGATGT	611
	3	487	*TGTAAAACGACGGCCAGT*CCTGGTCAYGCAGACCTCAG	1019	*CAGGAAACAGCTATGACC*CCAAARCTGAAGGAYGARCTGAT	569
	5	946	*TGTAAAACGACGGCCAGT*CCRACWGAAGAACAAGCTGT	1509	*CAGGAAACAGCTATGACC*GGAGTATTCATCYACACCCAT	600
	7	1447	*TGTAAAACGACGGCCAGT*CCAAGYACMGAGATGTCAATGAGA	2186	*CAGGAAACAGCTATGACC*TTRCTCARTTCATTGATGCT	776
	8	1683	*TGTAAAACGACGGCCAGT*CAACACTTATCAATGGATAAT	2341	*CAGGAAACAGCTATGACC*TAGTAGAAACAAGGTCGTT	695
PB1	1	22	*TGTAAAACGACGGCCAGT*AGCAAAAGCAGGCAAACCAT	534	*CAGGAAACAGCTATGACC*TCTATTAGCCTCCCWGAYTCATT	549
	3	389	*TGTAAAACGACGGCCAGT*ACAAGRGTGGACAAATRAC	1041	*CAGGAAACAGCTATGACC*CTGAACCAYTCAGGYTGATTT	689
	5	974	*TGTAAAACGACGGCCAGT*AATCAAAAYCCTMGAATGTT	1566	*CAGGAAACAGCTATGACC*AGCTCCATGCTRAAATTRGC	629
	7	1489	*TGTAAAACGACGGCCAGT*ATGAGYAAAAAGAAGTCYTA	1954	*CAGGAAACAGCTATGACC*TCAATYTCYTTATGGCTGAC	502
	8	1532	*TGTAAAACGACGGCCAGT*GCYAATTTYAGCATGGAGCT	2321	*CAGGAAACAGCTATGACC*AGTAGAAACAAGGCATTT	826
PA	1	0	*TGTAAAACGACGGCCAGT*AGCAAAAGCAGGTACTGAT	493	*CAGGAAACAGCTATGACC*TAGTCSGCCTTTGTGGCCATYTC	529
	3	361	*TGTAAAACGACGGCCAGT*TATGAYTACAARGAGAA	989	*CAGGAAACAGCTATGACC*GGTTCTTTCCATCCAAAGAATGTT	665
	5	894	*TGTAAAACGACGGCCAGT*AAATTRAGCATTGARGAYCCG	1662	*CAGGAAACAGCTATGACC*TCWAGTCTYGGGTCAGTGAG	805
	7	1444	*TGTAAAACGACGGCCAGT*AATGCATCCTGTGCAGCAATGGA	2057	*CAGGAAACAGCTATGACC*TTGTCCCTAAGAGCCTGAACAA	650
	8	1787	*TGTAAAACGACGGCCAGT*ATGAARTGGGGAATGGAGATGAG	2233	*CAGGAAACAGCTATGACC*AGTAGAAACAAGGTACCTTTT	483
HA	1	1	*TGTAAAACGACGGCCAGT*ATACGACTAGCAAAAGCAGGGG	461	*CAGGAAACAGCTATGACC*TCATGATTGGGCCAYGA	497
	2	351	*TGTAAAACGACGGCCAGT*ACRTGTTACCCWGGGRGATTTCA	943	*CAGGAAACAGCTATGACC*GAAAKGGGAGRCTGGTGTTTA	629
	4	736	*TGTAAAACGACGGCCAGT*AGRATGRACTATTACTGGAC	1340	*CAGGAAACAGCTATGACC*TTCTKCATTRTAWGTCCAAA	623
	6	1204	*TGTAAAACGACGGCCAGT*AAGATGAAYACRCARTTCACAG	1778	*CAGGAAACAGCTATGACC*GTGTCAGTAGAAACAAGGGTGTTT	593
NP	1	1	*TGTAAAACGACGGCCAGT*CAGGGTAGATAATCACTCAC	553	*CAGGAAACAGCTATGACC*AGAGCACATYCTGGGATCCAT	589
	3	513	*TGTAAAACGACGGCCAGT*TGGCATTCHAATTTRAATGAT	1042	*CAGGAAACAGCTATGACC*CTGRCTCTTGTGTGCDGG	566
	5	872	*TGTAAAACGACGGCCAGT*TTCTGAGRGGRTCAGTTGCTC	1565	*CAGGAAACAGCTATGACC*AGTAGAAACAAGGGTATTTTTC	730
NA	1	0	*TGTAAAACGACGGCCAGT*AGCAAAAGCAGGAGT	600	*CAGGAAACAGCTATGACC*CTGGACCRGAAATTCC	636
	2-3	318	*TGTAAAACGACGGCCAGT*TACACAAAAGACAAYAGC	1063	*CAGGAAACAGCTATGACC*CATATYTGTATGAAAACC	782
	4-5	726	*TGTAAAACGACGGCCAGT*AATGGRCARGCCTCRTACAA	1452	*CAGGAAACAGCTATGACC*AGTAGAAACAAGGAG	762
M	1	0	*TGTAAAACGACGGCCAGT*AGCAAAAGCAGGTAG	473	*CAGGAAACAGCTATGACC*GCAATCTGYTCACAKGT	509
	2	223	*TGTAAAACGACGGCCAGT*CACCGTGCCCAGTGAGCG	750	*CAGGAAACAGCTATGACC*TCAYTTGAAYCGYTGCAT	564
	3	383	*TGTAAAACGACGGCCAGT*TCTGCTGGWGCACTTGCCAGTTG	1027	*CAGGAAACAGCTATGACC*AGTAGMAACAAGGTAGT	681
NS	1	24	*TGTAAAACGACGGCCAGT*AGCAAAAGCAGGGTGACAAAGACA	482	*CAGGAAACAGCTATGACC*TCGGTGAAAGCCCTTA	495
	2	250	*TGTAAAACGACGGCCAGT*TGAGGCWYTTAAAATGACCA	890	*CAGGAAACAGCTATGACC*AGTAGAAACAAGGGTGTTTTTTAT	677

bp: base pairs

The M13-forward or M13-reverse universal primer sequence (18-nucleotide length) included in the sequence of each amplification primer is indicated in italic and dark grey.

Table S3.5 Primer set used for amplification and pyrosequencing of the influenza A virus M2 protein region associated with M2 inhibitor resistance (M gene) and of the A(H1N1)pdm09 and influenza B virus neuraminidase region including amino acid positions 275 (H275Y) and 197 (D197N), respectively.

Gene segment	Influenza virus type/subtype	PRIMER		Expected size amplicon (bp)
		Designation	Sequence (5´- 3´)	
M	A	M2 Forward	CAGATGCARCGATTCAGTG	264
		M2 Reverse *	AGTAGAAACAAGGTAGTTTTTTACTC	
		M2 Sequencing (forward)	CAGATGCAGCAATTCAGTG	-
NA	A(H1N1)pdm09	PanSwH1N1 275Forward *	GGGAAAGATAGTCAAATCAGTCGA	242
		PanSwH1N1 275Reverse	TAGACGATACTGGACCACAACTG	
		PanSwH1N1 275 Sequencing (reverse)	CAGGAGCATTCCTCA	-
	B	B 197-221 Forward	TTCAGTCAAATTGGGCAAAATC	242
		B 197-221 Reverse *	AGCTGGGCCATCAGTTATCATAAG	
		B 197-221 Sequencing (forward)	GGACATATATCGGAGTTG	-

bp: base pairs

The primers labelled with a biotin molecule at their 5´end are indicated with an asterisk (*). The direction of the sequencing primers is indicated under brackets after their designation.

CHAPTER 4

PHENOTYPIC AND/OR GENOTYPIC ANTIVIRAL SUSCEPTIBILITY PROFILES OF HUMAN INLUENZA VIRUSES CIRCULATING IN PORTUGAL

Most results published in:

Correia V, Santos LA, Gíria M, Santos MM, Rebelo-de-Andrade H, 2015. Influenza A(H1N1)pdm09 resistance and cross-reduced susceptibility to oseltamivir and zanamivir antiviral drugs. J Med Virol 87(1), 45-56.

Gíria M, Rebelo-de-Andrade H, Santos LA, **Correia V**, Pedro S, Santos MM, 2012. Genomic signatures and antiviral drug susceptibility profile of A(H1N1)pdm09. J Clin Virol 53(2), 140-144.

Santos LA, **Correia V**, Giria M, Pedro S, Santos MM, Silvestre MJ, Rebelo-de-Andrade H, 2011. Genetic and Antiviral Drug Susceptibility Profiles of Pandemic A(H1N1)v Influenza Virus Circulating in Portugal. Influenza Other Respi Viruses 5 (Suppl. 1), 294–300.

Correia V, Rebelo-de-Andrade H, Santos LA, Lackenby A, Zambon M, 2010. Antiviral drug profile of seasonal influenza viruses circulating in Portugal from 2004/2005 to 2008/2009 winter seasons. Antivir Res 86(2), 128-136.

Santos LA, **Correia V**, Pedro S, Alverca E, Santos MM, Silvestre MJ, Rebelo-de-Andrade H. 2010. Caracterização genética da nova variante pandémica do vírus influenza A(H1N1) 2009 em circulação em Portugal: resultados preliminares. RPDI 6(1), 7-13.

Correia V, Santos LA, Rebelo-de-Andrade H. 2009. Emergência de resistência aos antivirais específicos para a gripe em Portugal. RPDI 5(1), 17-24.

I carried out most activities, methodologies and data analysis underlying the results presented in this chapter. A low percentage (about 20%) of the influenza A(H1N1)pdm09 virus M gene sequences used for genotypic evaluation of M2 inhibitor susceptibility were obtained by a team colleague, in the context of the study developed for whole-genome characterization of the newly emerging pandemic virus. The phenotypic susceptibility profile of former seasonal A(H1N1), A(H3N2) and influenza B viruses from 2004/2005 to 2008/2009 to zanamivir was essentially determined by Mafalda Uva, in the context of her Master´s thesis in Applied Microbiology (Faculdade de Ciências, Universidade de Lisboa). A team colleague carried out the chemiluminescent NA inhibition assays necessary for confirming the fluorescent-based phenotypic susceptibility profile of a limited number of viruses from 2004/2005 to 2007/2008 to oseltamivir. The same colleague also sequenced the HA1 subunit of the HA protein of the phenotypic outlier viruses from 2004/2005 to 2008/2009. Some NA and HA sequences of non-outlier former seasonal A(H1N1), A(H3N2) and influenza B viruses from 2004/2005 to 2006/2007 (retrospective analysis) were available in the laboratory sequence database, having been obtained to study the evolutionary dynamics of the two major influenza virus antigens. The NA and HA sequences of the non-outlier A(H1N1)pdm09 viruses from 2009 to 2010/2011 were obtained by me and two other colleagues in the context of the study developed for whole-genome characterization of the pandemic virus. Lastly, the NA and HA sequences of the A(H1N1)pdm09 virus population present in the three respiratory specimens (one from before and two from after starting antiviral therapy) were obtained by João Louro in the context of his Master´s thesis in Pharmaceutical Sciences (Faculdade de Farmácia, Universidade de Lisboa). This sequencing work was performed at the Public Health England (Colindale, London, United Kingdom).

FCG-funded research projects

"*Vigilância e Monitorização da Susceptibilidade aos Antivirais Específicos para a Gripe*" (FCG 76676)

"*Avaliação e Caracterização da Emergência das Resistências aos Antivirais Específicos para a Gripe no Contexto da Infecção Respiratória Aguda*" (SDH49; ACSS reinforcement grant)

4 PHENOTYPIC AND/OR GENOTYPIC ANTIVIRAL SUSCEPTIBILITY PROFILES OF HUMAN INLUENZA VIRUSES CIRCULATING IN PORTUGAL

"Then there is the danger that the ignorant man may easily underdose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant."

Alexander Fleming, Penicillin, Nobel Lecture, December 11, 1945

In this chapter are presented and discussed the results of the antiviral susceptibility testing performed on human influenza viruses circulating in Portugal from 2004/2005 to 2012/2013 seasons, in both community and hospital settings (1st general objective; see section 2.2, Study Description). Virus susceptibility to M2 protein inhibitors (herein designated as M2 inhibitors; amantadine (AMA) and rimantadine (RIM)) was determined by genotypic testing, while a combined laboratorial approach involving phenotypic and genotypic testing was used to evaluate the susceptibility to the neuraminidase (NA) inhibitors (NAIs) oseltamivir (OS) and zanamivir (ZA).

The main activities carried out during this study are summarized below for a better understanding of the underlying work.

BOX 4.1 - MAIN ACTIVITIES

- Implementation of fluorescent MUNANA-based and chemiluminescent NA-Star® kit NA inhibition assays.
- Implementation of Sanger sequencing protocols targeting the M2 and HA coding regions of former seasonal A(H1N1), A(H3N2) and influenza B viruses (integrated in whole-genome sequencing protocol implementation).
- Isolation and/or propagation of human influenza viruses in MDCK or MDCK-SIAT1 cells.
- M2 gene pyrosequencing or Sanger sequencing of circulating human influenza A viruses, followed by screening of the 7 established molecular markers of M2 inhibitor resistance (L26F, V27A, A30T/V, S31N/D, G34E) in the M2 protein sequences.
- Determination of the OS and ZA IC_{50} values of circulating human influenza viruses by fluorescent MUNANA-based assay.
- Statistical determination of OS and ZA IC_{50} cut-offs, baseline median and fold-change thresholds, for each influenza virus type or subtype and influenza season/pandemic period.

BOX 4.1 - MAIN ACTIVITIES (cont.)

- Identification of the OS and ZA IC_{50} outlier viruses and of the viruses exhibiting (highly) reduced inhibition to OS and/or ZA *in vitro*.
- Confirmation of fluorescent-based NAI susceptibility phenotypes by chemiluminescent assay (carried out for a limited number of viruses).
- NA and HA gene sequencing of IC_{50} outlier viruses and of further viruses recovered from patients during or after antiviral therapy, and of ≈25% of the non-outlier type/subtype-matched viruses from the same influenza season/pandemic period.
- Identification of the NA and/or HA amino acid substitutions specific of IC_{50} outlier viruses and of further viruses recovered from patients during or after antiviral therapy, followed by their characterization regarding association to NAI resistance or decreased susceptibility and location onto the protein structure.
- Screening of the HA amino acid substitutions identified in influenza viruses recovered from patients under antiviral therapy or selected *in vitro* following serial passage under NAI drug pressure in all sequences obtained.
- Analysis of the evolutionary relationships among the NA and HA genes of IC_{50} outlier viruses and/or viruses recovered from patients during or after antiviral therapy and of non-outlier and worldwide reference viruses from the same influenza virus type or subtype.
- Analysis of the relationship between decreased NAI susceptibility phenotypes and NA genotypic background and, whenever possible, between these and patient clinical data.

4.1 RESULTS

The results are organized in 5 main sub-sections as follows: (1) technological platform for evaluation of antiviral susceptibility; (2) target population and study sample; (3) M2 inhibitor susceptibility testing; (4) oseltamivir and zanamivir susceptibility testing; and (5) antiviral prescription on influenza-like illness patients (complementary study).

4.1.1 Technological Platform for Evaluation of Antiviral Susceptibility

A technological platform for comprehensive evaluation of influenza virus susceptibility to the three antivirals licensed in Portugal for clinical use - AMA (M2 inhibitor) and OS and ZA (NAIs), was designed in late 2007 according to the approaches and methodologies recommended by the former European Surveillance Network for Vigilance against Viral

Resistance (VIRGIL) [1,2]. The platform was progressively established between 2008 and 2009 combining (1) newly implemented methodologies and/or protocols; (2) sequencing strategies and protocols available and in use in the laboratory to study the evolutionary dynamics of circulating influenza viruses; and (3) the state-of-the-art pyrosequencing technology available at the former Health Protection Agency (HPA) (Colindale, London, United Kingdom (UK), which is now part of Public Health England (PHE)), in the context of the close collaboration established with HPA in both research projects funding this work (project partner). Moreover, it was strengthened in 2012 by implementing a Sanger sequencing alternative to M2 pyrosequencing (M gene) and by expanding hemagglutinin (HA) gene sequencing to the entire coding region (previous protocol only targeted HA1 subunit). Over the last few years, minor but important updates have been performed in the platform, essentially at interpretation level, based on the recommendations and information issued by both the World Health Organization (WHO) Expert Working Group on Surveillance of Influenza Antiviral Susceptibility (AVWG) and the European Centre for Disease Prevention and Control (ECDC) Antiviral Susceptibility Task Group [3-5].

The platform includes a genotypic platform for M2 inhibitor susceptibility testing (complete cross-resistance between AMA and RIM [6]) and a twofold phenotypic-genotypic platform combined, whenever possible, with patient clinical data, for OS and ZA susceptibility testing (Figure 4.1). Two alternative sequencing methodologies are available for genotypic testing of M2 inhibitor susceptibility: (1) pyrosequencing of a short M gene region covering the 5 well-known amino acid sites in influenza A M2 protein associated with drug resistance (26, 27, 30, 31 and 34) (see section 3.2.1.4.3, Material and Methods); and (2) Sanger sequencing of the entire coding region of M2 protein (see RT-PCR virus genome amplification protocols in section 3.2.1.4.2.1, Material and Methods). Resistance is evaluated through the screening of the 7 established molecular markers of clinical resistance in M2 protein sequence (L26F, V27A, A30T/V, S31N/D, G34E). OS and ZA susceptibility testing is intended to cover the three different levels at which antiviral resistance can be defined: phenotypically, genetically and clinically. Two different NA inhibition assays comprise the phenotypic component of the platform: fluorescent MUNANA-based assay (see section 3.2.1.3.1, Material and Methods), used as reference assay; and chemiluminescence NA-Star® kit assay (see section 3.2.1.3.2, Material and Methods), used to confirm fluorescent-based results, particularly IC_{50} outlier values and whenever it is observed a significant difference between the

results of inter-assay virus replicates. NA inhibition assay data is used to determine the concentration of OS or ZA required to inhibit a standardised amount of virus NA activity by 50% (IC_{50} value) (detailed in section 3.2.2.1.1, Material and Methods). Further analysis of IC_{50} data, including determination of statistical cut-offs, baseline median (median without IC_{50} outlier values) and fold-change thresholds allow to assess the virus susceptibility to the drug (see section 3.2.2.1.3, Material and Methods). Phenotypic drug susceptibility profiles are defined according to the new inhibition categories established by the WHO AVWG: *normal inhibition* (NI) (fold-change increase <10 (influenza A) or <5 (influenza B)); *reduced inhibition* (RI) (fold-change increase 10-100 (influenza A) or 5-50 (influenza B)); *highly reduced inhibition* (HRI) (fold-change increase >100 (influenza A) or >50 (influenza B)) [4].

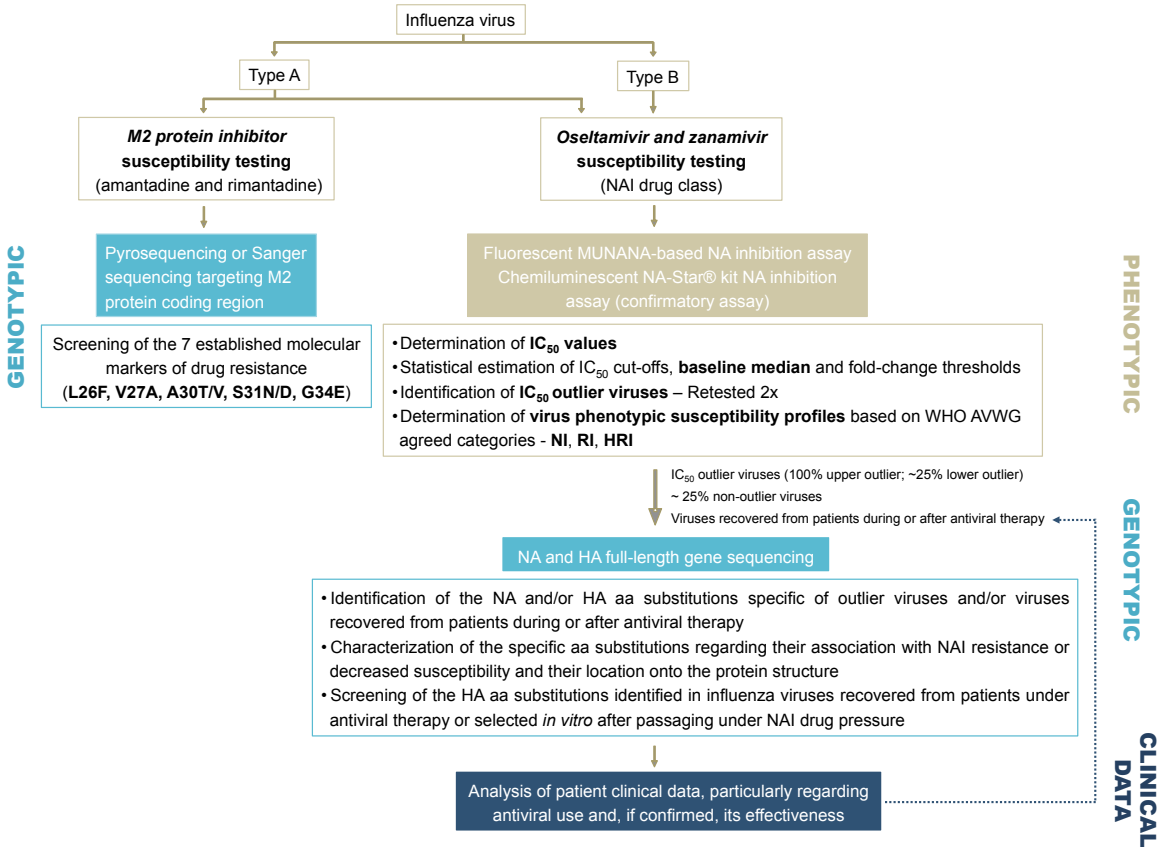


Figure 4.1 Schematic representation of the technological platform established for influenza antiviral susceptibility testing.

aa: amino acid; IC_{50}: Concentration of antiviral required to inhibit a standardised amount of virus NA activity by 50%; HA: Hemagglutinin; HRI: Highly reduced inhibition; NA: Neuraminidase; NAI: NA inhibitor; NI: Normal inhibition; RI: Reduced inhibition

IC_{50} outlier influenza viruses, including all upper outlier viruses and ≈25% of the lower outlier viruses, and ≈25% of the non-outlier viruses from the same influenza type or subtype and season (randomly selected), are further evaluated through genotypic testing (Figure 4.1). Influenza viruses recovered from patients during or after antiviral therapy but not classified as outlier or not possible to evaluate by phenotypic testing (unsuccessful virus isolation), also comprise this group, as well as the corresponding viruses from before therapy, whenever available.

Genotypic testing of NAI susceptibility involves both NA and HA full-length gene sequencing (Sanger method; see section 3.2.1.4.2, Material and Methods), using the cell passage isolate characterized by phenotypic assay. Clinical specimens are only used if related to antiviral therapy or to confirm the presence of a specific amino acid substitution in the original virus population (possibility of cell culture artefacts). The NA and/or HA amino acid substitutions specific of IC_{50} outlier viruses and of further viruses recovered from patients during or after antiviral therapy are then identified and characterized regarding their association to NAI resistance or decreased susceptibility (see Table 1.2, Literature Review for NA substitutions; HA D221G/N, seasonal H1 numbering) and their location onto the protein structure. NA amino acid substitutions at or near the active site may affect drug binding affinity and consequently virus susceptibility; while HA amino acid substitutions located at or near the receptor binding site (RBS) may change receptor binding affinity and disturb the HA-NA balance, which may result in altered virus fitness or even in altered virus susceptibility if it changes the requirements for NA activity (NA inhibition assay only accounts for NA-mediated susceptibility). Genotypic analysis of HA gene also comprises the screening of the amino acid substitutions identified in influenza viruses recovered from patients under antiviral therapy or selected *in vitro* after serial passage under NAI drug pressure (see section 1.5.2.1.2, Literature Review).

Patient clinical data is essential for evaluating the relationship of decreased phenotypic susceptibility and/or the presence of specific amino acid substitutions in NA and/or HA with NAI drug use and, particularly, its clinical effectiveness. However, comprehensive patient clinical data is usually difficult to obtain.

4.1.2 Target Population and Study Sample

Antiviral susceptibility testing was performed on human influenza viruses circulating in Portugal from 2004/2005 to 2012/2013 among both community and hospitalized patients, either or not under influenza antiviral therapy (target population). Influenza viruses from 2004/2005 to 2007/2008 were retrospectively studied to cover the first seasons for which was available information on the susceptibility of influenza viruses circulating in Europe to antiviral drugs, generated by the former VIRGIL network (2004 to 2008) [7].

Effective study sample comprised most influenza viruses selected for study (presented in section 3.1.1, Material and Methods). A total of 357 influenza A viruses (84 former seasonal A(H1N1), 142 A(H3N2) and 131 A(H1N1)pdm09) were tested for M2 inhibitor susceptibility, while a total of 526 (93 former seasonal A(H1N1), 144 A(H3N2), 142 B, 147 A(H1N1)pdm09) and 491 (76 former seasonal A(H1N1), 139 A(H3N2), 129 B, 147 A(H1N1)pdm09) influenza viruses were tested for, respectively, OS and ZA susceptibility (Table 4.1). Most influenza viruses were tested using a 2nd to 4th cell passage isolate.
The percentage of A(H3N2) and A(H1N1)pdm09 viruses tested for M2 inhibitor susceptibility decreased significantly after 2008/2009 and the 2009 pandemic period, respectively, given the overall resistance observed to this antiviral drug class. Virtually all viruses not evaluated through OS or ZA phenotypic susceptibility testing had issues of either not enough volume of clinical specimen for virus isolation or unsuccessful virus isolation or propagation in cell culture. Since ZA susceptibility was only tested after OS susceptibility until 2008/2009, occasional further propagation in cell culture was needed and sometimes was unsuccessful, explaining the lower number of viruses evaluated against ZA (Table 4.1). The influenza viruses evaluated through NA and/or HA gene sequencing included the viruses that were predetermined to be further evaluated by genotypic NAI susceptibility, with the few exceptions being described below in section 4.1.4.2. It also included additional viruses (non-outlier) for which the NA and/or HA sequences were obtained in the context of the on-going laboratory study on the evolutionary dynamics of the two major influenza virus antigens. These additional sequences increased the robustness of both genetic and phylogenetic comparative analyses performed.

Table 4.1 Number (percentage) of influenza viruses evaluated by each genotypic and/or phenotypic antiviral susceptibility testing performed to assess M2 inhibitor and oseltamivir and zanamivir neuraminidase inhibitor susceptibility.

Influenza virus type/subtype	Influenza season/ 2009 pandemic period [a]	Number of viruses selected for study	M2 inhibitor susceptibility testing - Genotypic [b]	NAI susceptibility testing			
				Phenotypic (MUNANA-based assay) [b]		Genotypic [c]	
				Oseltamivir	Zanamivir	NA sequencing	HA sequencing
Former seasonal A(H1N1) [d]	2004/2005	6	6 (100.0)	4 (66.7)	3 (50.0)	2 (50.0)	2 (50.0)
	2005/2006	46	45 (97.8)	39 (84.8)	27 (58.7)	11 (28.2)	12 (30.8)
	2006/2007	-[e]	-	-	-	-	-
	2007/2008	29	18 (62.1)	29 (100.0)	28 (96.6)	23 (79.3)	15 (51.7)
	2008/2009	21	15 (71.4)	21 (100.0)	18 (85.7)	21 (100.0)	11 (52.4)
	Pandemic period	2	0 (0.0)	0 (0.0)	0 (0.0)	-[f]	-[f]
	TOTAL	104	84 (80.8)	93 (89.4)	76 (73.1)	57 (61.3)	40 (43.0)
A(H3N2)	2004/2005	89	76 (85.4)	76 (85.4)	71 (79.8)	20 (26.3)	19 (25.0)
	2005/2006	1	1 (100.0)	1 (100.0)	1 (100.0)	1 (100.0)	1 (100.0)
	2006/2007	76	51 (67.1)	20 (26.3)	20 (26.3)	14 (70.0)	12 (60.0)
	2007/2008	-[e]	-	-	-	-	-
	2008/2009	21	10 (47.6)	20 (95.2)	20 (95.2)	14 (70.0)	14 (70.0)
	Pandemic period	5	0 (0.0)	0 (0.0)	0 (0.0)	-[f]	-[f]
	2010/2011	2	0 (0.0)	0 (0.0)	0 (0.0)	-[f]	-[f]
	2011/2012	30	3 (10.0)	26 (86.7)	26 (86.7)	9 (34.6)	9 (34.6)
	2012/2013	1	1 (100.0)	1 (100.0)	1 (100.0)	1 (100.0)	1 (100.0)
	TOTAL	225	142 (63.1)	144 (64.0)	139 (61.8)	59 (41.0)	56 (38.9)
B	2004/2005	35	-[g]	33 (94.3)	24 (68.6)	4 (12.1)	13 (39.4)
	2005/2006	49	-[g]	49 (100.0)	47 (95.9)	4 (8.2)	13 (26.5)
	2006/2007	2	-[g]	2 (100.0)	2 (100.0)	2 (100.0)	2 (100.0)
	2007/2008	45	-[g]	45 (100.0)	43 (95.6)	18 (40.0)	12 (26.7)
	2008/2009	1	-[g]	1 (100.0)	1 (100.0)	1 (100.0)	1 (100.0)
	Pandemic period	10	-[g]	3 (30.0)	3 (30.0)	3 (100.0)	2 (66.7)
	2010/2011	5	-[g]	2 (40.0)	2 (40.0)	2 (100.0)	2 (100.0)
	2011/2012	2	-[g]	1 (50.0)	1 (50.0)	1 (100.0)	1 (100.0)
	2012/2013	9	-[g]	6 (66.7)	6 (66.7)	3 (50.0)	3 (50.0)
	TOTAL	158	-[g]	142 (89.9)	129 (81.6)	38 (26.8)	49 (34.5)
A(H1N1)pdm09	Pandemic period	163	116 (71.2)	116 (71.2)	116 (71.2)	44 (37.9)	44 (37.9)
	2010/2011	69	13 (18.8)	26 (37.7)	26 (37.7)	13 (50.0)	13 (50.0)
	2011/2012	-[e]	-	-	-	-	-
	2012/2013	23	2 (8.7)	5 (21.7)	5 (21.7)	3 (60.0)	3 (60.0)
	TOTAL	255	131 (51.4)	147 (57.6)	147 (57.6)	60 (40.8)	60 (40.8)
TOTAL		***742***	***357 (61.1[h])***	***526 (70.9)***	***491 (66.2)***	***214 (40.7)***	***205 (39.0)***

HA: Hemagglutinin; NA: Neuraminidase; NAI: Neuraminidase Inhibitor

[a] Period between 11th June 2009 and 9th August 2010 (WHO pandemic alert Phase 6); [b] Percentage estimates based on the number of influenza viruses selected for study; [c] Percentage estimates based on the number of influenza viruses evaluated through phenotypic oseltamivir susceptibility testing; [d] No former seasonal A(H1N1) viruses were detected in Portugal after the pandemic 2009 period; [e] No influenza virus was detected during this influenza season; [f] No influenza virus was evaluated through phenotypic susceptibility testing; [g] Influenza B viruses are naturally resistant to M2 inhibitors (lack of M2 target protein); [h] Percentage of the overall influenza A viruses selected for study (N=593).

The viruses from 16 of the 30 cases of influenza virus infection associated with NAI drug use (detailed in section 3.1.1, Material and Methods) were among the total viruses evaluated to M2 inhibitor and both OS and ZA susceptibility. However, only the viruses

from 3 cases were effectively recovered from the patient after initiation of antiviral therapy. Specifically, the viruses from two of the cases of A(H1N1)pdm09 virus infection received with a high level of suspicion of clinical resistance to OS (case 1, A/Portugal/28/2009 virus isolate; and case 3, A/Portugal/03/2011 virus isolate; see Table 3.2, Material and Methods); and a virus from a case of A(H3N2) virus infection from 2012/2013 (A/Portugal/56/2013 virus isolate). The NA and HA genes of the virus population present in the clinical specimens yielding A/Portugal/28/2009 and A/Portugal/56/2013 isolates were also sequenced (not included in the numbers presented at Table 4.1). The same was not performed for virus isolate A/Portugal/03/2011 as preliminary HCC results had already identify NA H275Y OS-resistant marker in the entire virus population of the specimen (see Table 3.2, Material and Methods). NA and HA gene sequencing was also performed on two post-treatment clinical specimens (from during and after drug use) from an additional case of A(H1N1)pdm09 virus infection (2012/2013), for which virus isolation was only successfully performed for the clinical specimen collected before drug use (A/Portugal/55/2013 isolate) (also not taken into consideration for Table 4.1).

The viruses from the two other cases of A(H1N1)pdm09 virus infection suspected of clinical resistance to OS (case 2 and 4; see Table 3.2, Material and Methods), were not possible to study (unsuccessful virus isolation). But, for the latter case (case 4), it was possible to quantify the mixed NA H275Y/H virus population by pyrosequencing, using the RNA extracted material available – 73.8% wild-type H275, 26.2% mutant H275Y (data not shown; quantitation performed at the PHE, Colindale, London, UK).

4.1.3 M2 Inhibitor Susceptibility Testing

Resistance to M2 inhibitors was found in A(H3N2) and A(H1N1)pdm09 subtypes. An overall resistant profile was observed for A(H1N1)pdm09 subtype, with all 131 viruses tested harboring the M2 S31N resistant marker (naturally-resistant viruses), while the profile of circulating A(H3N2) viruses shifted from susceptible to resistant during the time period analyzed (Figure 4.2). All A(H3N2) viruses from 2004/2005 (n=76) were susceptible to this antiviral drug class. The single A(H3N2) virus from 2005/2006 was the first to exhibit drug resistance, followed by 13 (74.5%) of the 51 viruses from 2006/2007. An overall resistance (100.0%) was observed in 2008/2009, 2011/2012 and

2012/2013 (both pandemic period and 2010/2011 with no virus were tested) (Figure 4.2). The frequency of M2 inhibitor resistance among circulating A(H3N2) viruses was of approximately 37% for the total time period (2004/2005 to 2012/2013).

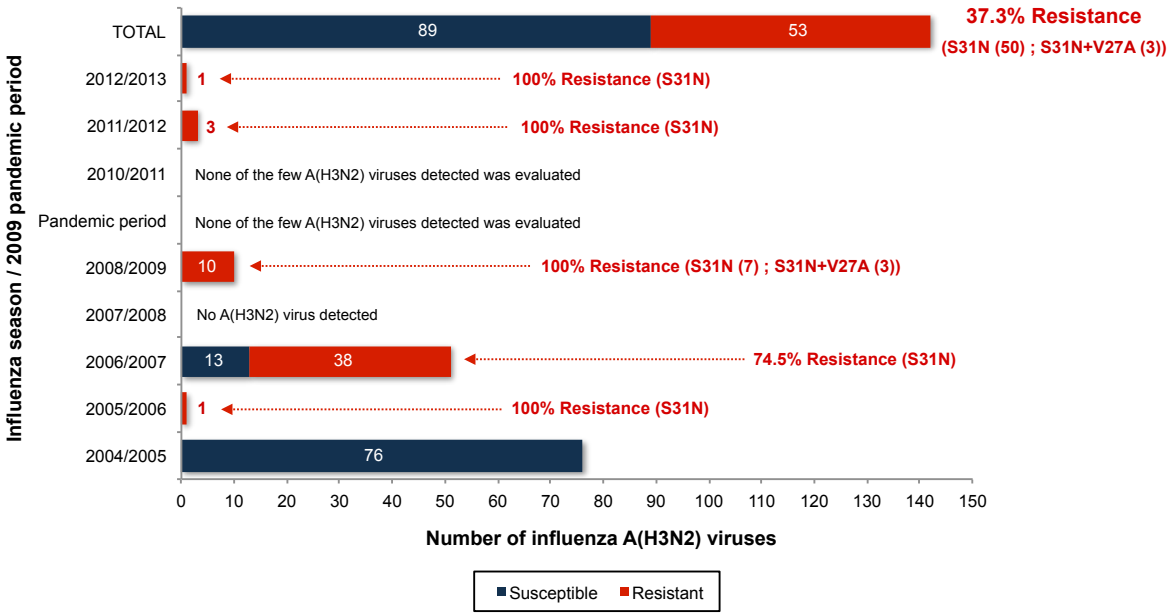


Figure 4.2 Susceptibility profile of circulating influenza A(H3N2) viruses to M2 protein inhibitors.
The resistant molecular marker(s) found in the M2 protein sequences are indicated within brackets after the resistance frequency.

All A(H3N2) resistant viruses carried the S31N amino acid substitution in the M2 protein, with three (5.7%, 3/53) viruses from 2008/2009 further carrying the V27A substitution (double V27A/S31N mutant viruses) (Figure 4.3).

All former seasonal A(H1N1) viruses (herein designated as seasonal A(H1N1)) showed to be susceptible to M2 inhibitors. None of the established molecular markers of resistance was found in the M2 protein sequences of the total 84 seasonal A(H1N1) viruses analysed.

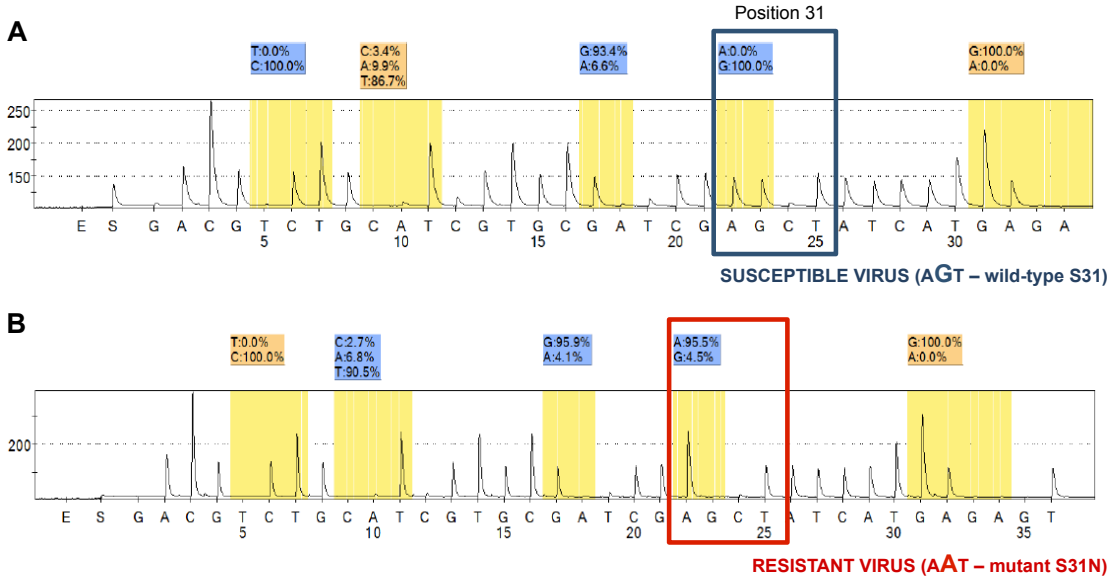


Figure 4.3 Representative M2 gene pyrograms of influenza A(H3N2) viruses susceptible (wild-type) (**A**) and resistant (S31N) (**B**) to M2 protein inhibitors.

The figure displays the pyrograms obtained for the A/Lisboa/14/2007 (A) and A/Lisboa/01/2007 (B) A(H3N2) virus isolates from 2006/2007. Pyrograms were obtained through SNP (single nucleotide polymorphism) pyrosequencing analysis using the PyroMark® Q96 ID (QIAGEN) platform. Yellow areas indicate the 5 positions at which the SNPs that cause M2 inhibitor resistance can occur (positions 26, 27, 30, 31 and 34 of the M2 protein). At the top of each position is given the percentage of wild-type (susceptible) versus mutant (resistant) virus. A limit of 10% was considered for resistance detection. The nucleotide change from G to A that underlays the resistant S31N SNP is highlighted in the figure. The pyrogram of the A(H3N2) susceptible virus (A) shows the sequence AC CCG CTT GTT GTT GTT GCC GCG **AGT** ATC ATT GGG AT, while the resistant S31N sequence (B) is AC CCG CTT GTT GTT GTT GCC GCG **AAT** ATC ATT GGG AT.

4.1.4 Oseltamivir and Zanamivir Susceptibility Testing

4.1.4.1 Phenotypic Testing

4.1.4.1.1 IC_{50} outlier viruses and virus phenotypic susceptibility profiles

4.1.4.1.1.1 Oseltamivir

All or nearly all (84.6% to 97.4%) influenza viruses from the different types or subtypes and influenza seasons or 2009 pandemic period, exhibited an IC_{50} value within the normal range (range of values between the lower and upper mild cut-offs) - non-outlier viruses (Table 4.2). Only for a single season within each influenza type or subtype this was not observed, with the frequency of non-outlier viruses being comparatively lower.

Specifically, for 2007/2008 in seasonal A(H1N1) (62.1%), 2006/2007 in A(H3N2) (70.0%), 2010/2011 in influenza B (50.0%), and 2012/2013 in A(H1N1)pdm09 (20.0%) type/subtype. Also, none of the seasonal A(H1N1) and influenza B viruses from 2008/2009 were non-outlier. IC_{50} value ranges of non-outlier viruses can be found at Table 4.2.

Table 4.2 Summary of phenotypic oseltamivir IC_{50} outlying results.

Influenza virus type/subtype	OSELTAMIVIR										
	Influenza season / 2009 pandemic period [a]	Number of viruses tested	NON-OUTLIER VIRUSES		OUTLIER VIRUSES - Number (%)						
			Number (%)	IC_{50} value range (nM)	UPPER			LOWER			***TOTAL***
					Mild	Extreme	***TOTAL***	Mild	Extreme	TOTAL	
Former seasonal A(H1N1)	2004/2005	4	4 (100.0)	1.63 - 2.13	0 (0.0)	0 (0.0)	*0 (0.0)*	0 (0.0)	0 (0.0)	0 (0.0)	*0 (0.0)*
	2005/2006	39	37 (94.9)	0.88 - 2.60	1 (2.6)	0 (0.0)	***1 (2.6)***	1 (2.6)	0 (0.0)	1 (2.6)	***2 (5.1)***
	2006/2007	-[b]	-	-	-	-	-	-	-	-	-
	2007/2008	29	18 (62.1)	1.53 - 2.60	1 (3.4)	6 (20.7)	***7 (24.1)***	3 (10.3)	1 (3.4)	4 (13.8)	***11 (37.9)***
	2008/2009	21	0 (0.0)	-	0 (0.0)	21 (100.0)	***21 (100.0)***	0 (0.0)	0 (0.0)	0 (0.0)	***21 (100.0)***
	Pandemic period	0	-	-	-	-	-	-	-	-	-
	TOTAL	93	59 (63.4)	0.88 - 2.60	2 (2.2)	27 (29.0)	***29 (31.2)***	4 (4.3)	1 (1.1)	5 (5.4)	***34 (36.6)***
A(H3N2)	2004/2005	76	74 (97.4)	0.22 - 0.61	0 (0.0)	0 (0.0)	*0 (0.0)*	2 (2.6)	0 (0.0)	2 (2.6)	***2 (2.6)***
	2005/2006	1	1 (100.0)	0.24	0 (0.0)	0 (0.0)	*0 (0.0)*	0 (0.0)	0 (0.0)	0 (0.0)	*0 (0.0)*
	2006/2007	20	14 (70.0)	0.35 - 0.47	1 (5.0)	1 (5.0)	***2 (10.0)***	2 (10.0)	2 (10.0)	4 (20.0)	***6 (30.0)***
	2007/2008	-[b]	-	-	-	-	-	-	-	-	-
	2008/2009	20	20 (100.0)	0.28 - 0.54	0 (0.0)	0 (0.0)	*0 (0.0)*	0 (0.0)	0 (0.0)	0 (0.0)	*0 (0.0)*
	Pandemic period	0	-	-	-	-	-	-	-	-	-
	2010/2011	0	-	-	-	-	-	-	-	-	-
	2011/2012	26	22 (84.6)	0.61 - 1.15	1 (3.8)	1 (3.8)	***2 (7.7)***	2 (7.7)	0 (0.0)	2 (7.7)	***4 (15.4)***
	2012/2013	1	1 (100.0)	1.01	0 (0.0)	0 (0.0)	*0 (0.0)*	0 (0.0)	0 (0.0)	0 (0.0)	*0 (0.0)*
	TOTAL	144	132 (91.7)	0.22 - 1.15	2 (1.4)	2 (1.4)	***4 (2.8)***	6 (4.2)	2 (1.4)	8 (5.6)	***12 (8.3)***
B	2004/2005 (YAM)	33	31 (93.9)	9.7 - 19.39	1 (3.0)	0 (0.0)	***1 (3.0)***	1 (3.0)	0 (0.0)	1 (3.0)	***2 (6.1)***
	2005/2006 (VIC)	49	44 (89.8)	16.72 - 26.72	2 (4.1)	0 (0.0)	***2 (4.1)***	3 (6.1)	0 (0.0)	3 (6.1)	***5 (10.2)***
	2006/2007	2	2 (100.0)	14.19 - 23.45	0 (0.0)	0 (0.0)	*0 (0.0)*	0 (0.0)	0 (0.0)	0 (0.0)	*0 (0.0)*
	2007/2008 (YAM)	45	45 (100.0)	11.9 - 33.61	0 (0.0)	0 (0.0)	*0 (0.0)*	0 (0.0)	0 (0.0)	0 (0.0)	*0 (0.0)*
	2008/2009 (VIC)	1	0 (0.0)	-	1 (100.0)	0 (0.0)	***1 (100.0)***	0 (0.0)	0 (0.0)	0 (0.0)	***1 (100.0)***
	Pandemic period (VIC)	3	3 (100.0)	44.22 - 69.14	0 (0.0)	0 (0.0)	*0 (0.0)*	0 (0.0)	0 (0.0)	0 (0.0)	*0 (0.0)*
	2010/2011	2	1 (50.0)	23.57	1 (50.0)	0 (0.0)	***1 (50.0)***	0 (0.0)	0 (0.0)	0 (0.0)	***1 (50.0)***
	2011/2012 (YAM)	1	1 (100.0)	21.59	0 (0.0)	0 (0.0)	*0 (0.0)*	0 (0.0)	0 (0.0)	0 (0.0)	*0 (0.0)*
	2012/2013 (YAM)	6	6 (100.0)	16.54 - 35.86	0 (0.0)	0 (0.0)	*0 (0.0)*	0 (0.0)	0 (0.0)	0 (0.0)	*0 (0.0)*
	TOTAL	142	133 (93.7)	9.7 - 69.14	5 (3.5)	0 (0.0)	***5 (3.5)***	4 (2.8)	0 (0.0)	4 (2.8)	***9 (6.3)***
A(H1N1)pdm09	Pandemic period	116	104 (89.7)	0.50 - 1.07	3 (2.6)	3 (2.6)	***6 (5.2)***	5 (4.3)	1 (0.9)	6 (5.2)	***12 (10.3)***
	2010/2011	26	23 (88.5)	0.69 - 1.21	2 (7.7)	1 (3.8)	***3 (11.5)***	0 (0.0)	0 (0.0)	0 (0.0)	***3 (11.5)***
	2011/2012	-[b]	-	-	-	-	-	-	-	-	-
	2012/2013	5	1 (20.0)	0.91	2 (40.0)	2 (40.0)	***4 (80.0)***	0 (0.0)	0 (0.0)	0 (0.0)	***4 (80.0)***
	TOTAL	147	128 (87.1)	0.50 - 1.21	7 (4.8)	6 (4.1)	***13 (8.8)***	5 (3.4)	1 (0.7)	6 (4.1)	***19 (12.9)***

VIC: Victoria; YAM: Yamagata

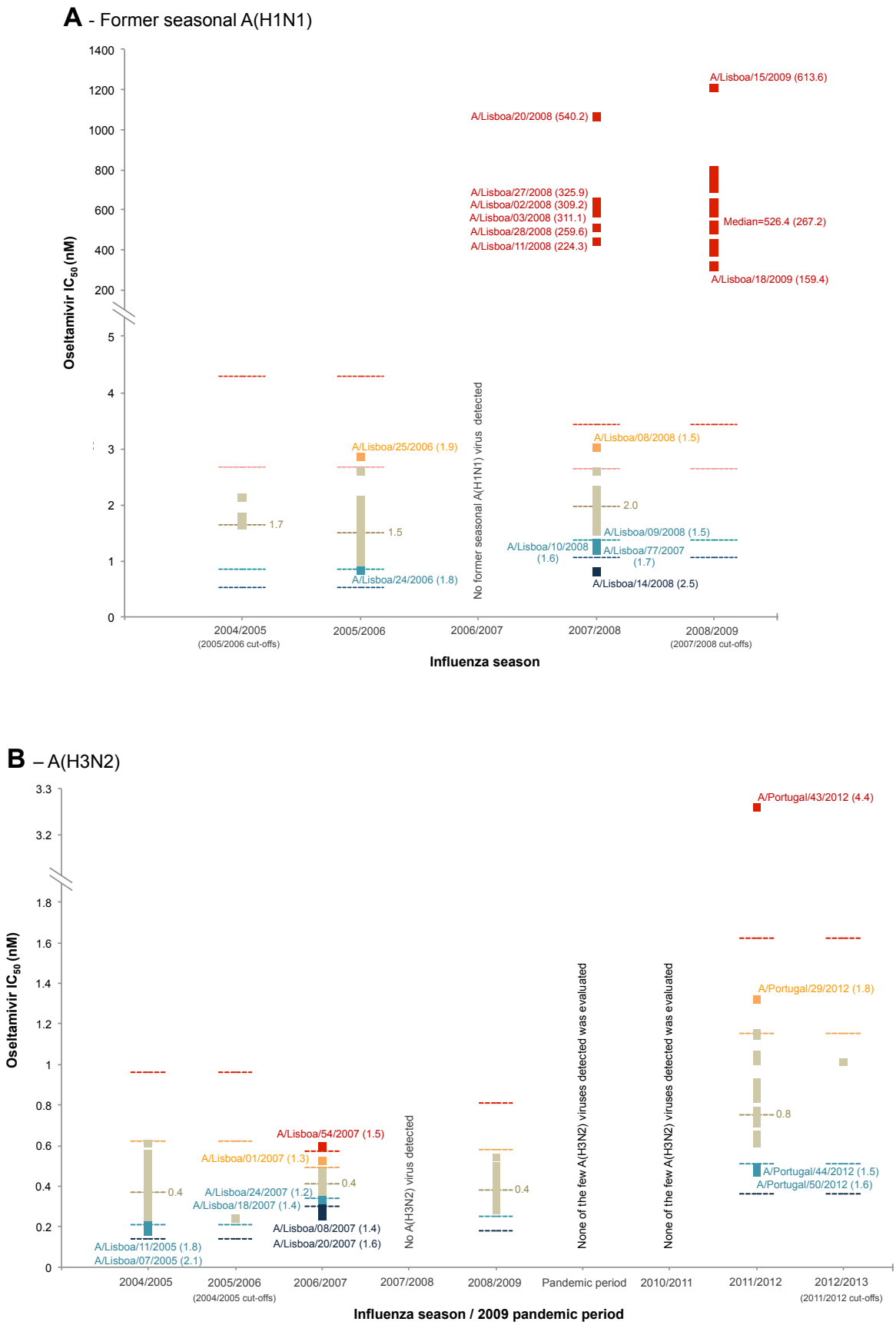
[a] The predominant influenza B lineage is indicated within brackets after the designation of the time period (lineage differentiation based on the antigenic and/or genetic characteristics of the virus hemagglutinin); [b] No influenza virus was detected.

These results were obtained through statistical analysis of the phenotypic IC_{50} data generated by fluorescent MUNANA-based neuraminidase inhibition assay. Non-outlier IC_{50} values are located between the lower and upper mild cut-offs (normal range). Upper outlier viruses had an IC_{50} value above mild (median+1.65 Standard Deviations (SD)) or extreme (median+3SD) upper cut-offs, while lower outlier viruses exhibited an IC_{50} value below mild (median-1.65SD) or extreme (median-3SD) lower cut-offs. The total number of upper and total outlier viruses is highlighted in bold and italic.

The overall range of IC_{50} non-outlier values was very similar between A(H3N2) (0.22 - 1.15nM) and A(H1N1)pdm09 (0.50 - 1.21nM) subtypes, varying from 0.88 to 2.60nM in seasonal A(H1N1) viruses. Influenza B non-outlier viruses exhibited the highest IC_{50} values that overall ranged from 9.7 to 69.14nM. The three influenza B non-outlier viruses from the pandemic period presented IC_{50} values considerably higher (44.22 - 69.14nM) than those yielded by all other non-outlier viruses (11.9 - 35.86nM). But, the use of pandemic period – 2012/2013 combined cut-offs, due to the low number of viruses tested, may be the reason why such viruses were not classified as outlier (see Figure 4.4C).

IC_{50} outlier viruses belonged to all 4 categories - upper mild (UM), upper extreme (UE), lower mild (LM), and lower extreme (LE). The only exception was in type B influenza, in which only mild outlier viruses were identified (UM and LM). Considering its distribution across the different categories, outlier viruses were detected at frequencies that ranged from 0.7% to 4.8% (overall frequencies), except seasonal A(H1N1) UE outlier viruses that represented nearly 30.0% of the viruses tested (Table 4.2). Additional information on OS IC_{50} outlier viruses is presented at Figure 4.4 A-D, in which are also indicated the IC_{50} cut-offs and baseline median (median without outlier values).

The frequency of seasonal A(H1N1) IC_{50} outlier viruses increased throughout influenza seasons. Initially estimated at 0.0% (2004/2005), it increased to 5.1% in 2005/2006 and then to 37.9% in 2007/2008, reaching 100% in 2008/2009 (Table 4.2). Most outlier viruses from 2007/2008 (n=6; 20.7%) and all outlier viruses from 2008/2009 (n=21), were UE outliers, exhibiting extremely high OS IC_{50} values that were ≈160 to 615-fold higher than the baseline median for 2007/2008 (1.97nM) (Figure 4.4A). The two seasonal A(H1N1) UM outlier viruses identified - virus isolates A/Lisboa/25/2006 (2005/2006) and A/Lisboa/08/2008 (2007/2008), presented an IC_{50} value ≈2-fold higher than the corresponding baseline median. The fold decrease in IC_{50} relative to baseline median exhibited by the 5 seasonal A(H1N1) lower outlier viruses (4 LM and 1 LE) was also very small (≤2-fold) (Figure 4.4A)


A - Former seasonal A(H1N1)
Oseltamivir IC50 (nM)
1400
1200
1000
800
600
400
200
5
4
3
2
1
0
A/Lisboa/15/2009 (613.6)
A/Lisboa/20/2008 (540.2)
A/Lisboa/27/2008 (325.9)
A/Lisboa/02/2008 (309.2)
A/Lisboa/03/2008 (311.1)
A/Lisboa/28/2008 (259.6)
A/Lisboa/11/2008 (224.3)
Median=526.4 (267.2)
A/Lisboa/18/2009 (159.4)
A/Lisboa/25/2006 (1.9)
1.7
1.5
A/Lisboa/24/2006 (1.8)
No former seasonal A(H1N1) virus detected
A/Lisboa/08/2008 (1.5)
2.0
A/Lisboa/09/2008 (1.5)
A/Lisboa/10/2008 (1.6)
A/Lisboa/77/2007 (1.7)
A/Lisboa/14/2008 (2.5)
2004/2005
(2005/2006 cut-offs)
2005/2006
2006/2007
2007/2008
2008/2009
(2007/2008 cut-offs)
Influenza season
B – A(H3N2)
Oseltamivir IC50 (nM)
3.3
3.2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
A/Portugal/43/2012 (4.4)
A/Portugal/29/2012 (1.8)
0.8
A/Portugal/44/2012 (1.5)
A/Portugal/50/2012 (1.6)
A/Lisboa/54/2007 (1.5)
A/Lisboa/01/2007 (1.3)
0.4
A/Lisboa/24/2007 (1.2)
A/Lisboa/18/2007 (1.4)
A/Lisboa/08/2007 (1.4)
A/Lisboa/20/2007 (1.6)
A/Lisboa/11/2005 (1.8)
A/Lisboa/07/2005 (2.1)
0.4
0.4
No A(H3N2) virus detected
None of the few A(H3N2) viruses detected was evaluated
None of the few A(H3N2) viruses detected was evaluated
2004/2005
2005/2006
(2004/2005 cut-offs)
2006/2007
2007/2008
2008/2009
Pandemic period
2010/2011
2011/2012
2012/2013
(2011/2012 cut-offs)
Influenza season / 2009 pandemic period

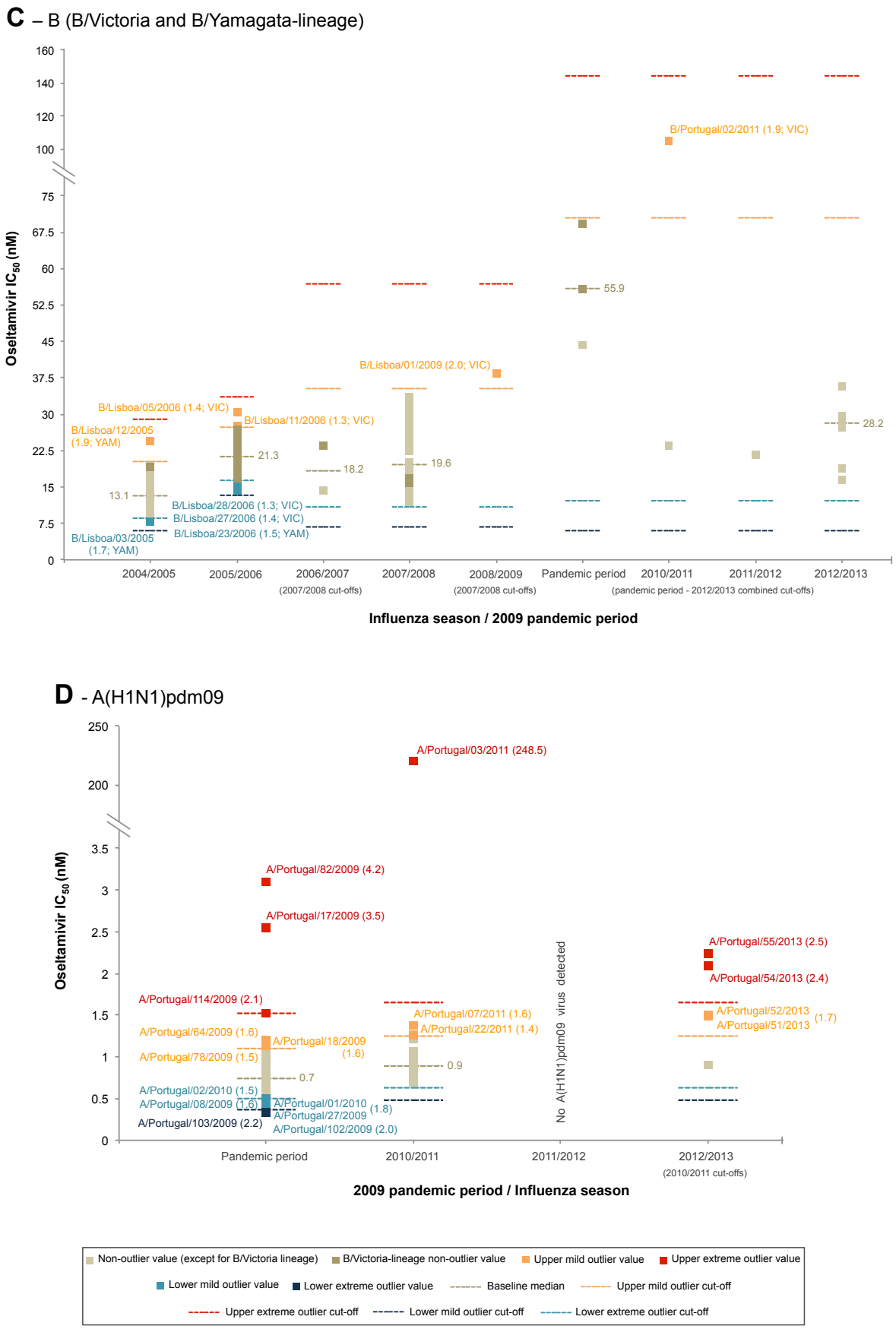


Figure 4.4 Oseltamivir IC_{50} values of circulating former seasonal A(H1N1) (**A**), A(H3N2) (**B**), influenza B (**C**) and A(H1N1)pdm09 (**D**) viruses.

(Footnotes Figure 4.4)

Mild outlier cut-offs were established at 1.65 standard deviations (SD) above (upper) or below (lower) the median, while a 3SD was considered for extreme cut-offs. IC_{50} values are displayed in different colours according to their outlier status and category (non-outlier, upper mild/upper extreme/lower mild/lower extreme outlier). An additional colour was used in the IC_{50} plot of influenza B viruses (plot C) to differentiate the non-outlier IC_{50} values of B/Victoria and B/Yamagata-lineage viruses (lineage differentiation based on the antigenic and/or genetic characteristics of the virus hemagglutinin). IC_{50} outlier cut-offs and baseline median (median without outlier values) are displayed using the same colour-coded system used for IC_{50} values. The designation of each outlier virus is indicated near the corresponding IC_{50} data point, followed by the fold-change IC_{50} increase/decrease compared to the baseline median, indicated within brackets. The influenza B lineage to which the outlier virus belongs is also indicated inside the brackets (exclusive of plot C). The IC_{50} baseline median value is displayed alongside with the marker. The IC_{50} cut-offs that were occasionally used for analysing IC_{50} values from previous or following influenza seasons for which less than 15 different viruses were tested are indicated in the x-axis, below the season of the analysed values. Pandemic period - 2012/2013 combined cut-offs were used for type B influenza (plot C), as the 12 different viruses from these 4-consecutive time periods were tested together.

A(H3N2) OS IC_{50} outlier viruses were only detected in 2004/2005 (n=2; 2.6%), 2006/2007 (n=6; 30.0%), and 2011/2012 (n=4; 15.4%), with half (6/12) of them belonging to the LM outlier category (Table 4.2). The two UM outlier viruses identified - virus isolates A/Lisboa/01/2007 (2006/2007) and A/Portugal/29/2012 (2011/2012), and the UE outlier virus from 2006/2007 (virus isolate A/Lisboa/54/2007) exhibited a ≤2-fold higher OS IC_{50} value, compared to the corresponding baseline median (Figure 4.4B). The UE outlier virus from 2011/2012 (virus isolate A/Portugal/43/2012) presented an IC_{50} value 4-fold higher than baseline median. All 8 lower outlier viruses (6 LM and 2 LE), presented a ≤2-fold decrease in IC_{50} relative to baseline median (Figure 4.4B).

Influenza B OS IC_{50} outlier viruses were detected in 2004/2005 (n=2; 6.1%), 2005/2006 (n=5; 10.2%), 2008/2009 (n=1; 100.0%), and 2010/2011 (n=1; 50.0%), distributing almost equitably between the two mild outlier categories – UM (n=5); and LM (n=4) (Table 4.2). A ≤2-fold increase or decrease in IC_{50} relative to baseline median was, respectively, observed for all UM and LM outlier viruses (Figure 4.4C). All influenza B UM outlier viruses belong to the B/Victoria (B/VIC) lineage, with exception of the virus isolate B/Lisboa/12/2005 from 2004/2005 (B/Yamagata (B/YAM) lineage). Due to the very limited number of viruses from the non-predominant B lineage in each influenza season or pandemic period, the IC_{50} values exhibited by the viruses from each lineage could not be analysed separately. However, as it is possible to observe in Figure 4.4C, the non-outlier IC_{50} values exhibited by B/VIC-lineage viruses tended to be higher than those from B/YAM-lineage viruses. Only for 2007/2008 this was not observed.

The frequency of A(H1N1)pdm09 OS IC_{50} outlier viruses was similar for the pandemic period (10.3%) and the 2010/2011 season (11.5%), but then increased greatly in 2012/2013 (80.0%)(Table 4.2). This increase may, however, be simply an artefact of the analysis of the few IC_{50} values of this season against the outlier cut-offs estimated for 2010/2011. Most A(H1N1)pdm09 outlier viruses were upper outlier, belonging to either UM (n=7; 4.8%) or UE (n=6; 4.1%) categories (Table 4.2). The UE outlier virus detected in 2010/2011 - virus isolate A/Portugal/03/2011, exhibited an extremely high OS IC_{50} value (219.95nM) that was ≈250-fold higher than the baseline median (Figure 4.4D). The two UE outlier viruses from the pandemic period – virus isolates A/Portugal/17/2009 and A/Portugal/82/2009, exhibited IC_{50} values, respectively, 3 and 4-fold higher than the baseline median. All remaining upper outlier viruses (3 UE, and 7 UM) presented ≤2-fold higher OS IC_{50} values. An ≈2-fold decrease in IC_{50} relative to baseline median was observed for all A(H1N1)pdm09 lower outlier viruses (5 LM, and 1LE) (Figure 4.4D).

Phenotypic drug susceptibility profiles were defined according to the WHO AVWG IC_{50} fold-change criteria, as detailed in section 4.1.1. Based on these criteria, 27 (29.0%) of the 93 seasonal A(H1N1) viruses showed HRI by OS (≈160 to 615-fold higher IC_{50}), corresponding to the 27 UE outlier viruses identified (6 from 2007/2008; 21 from 2008/2009) (Figure 4.4A). One (0.7%) A(H1N1)pdm09 virus from 2010/2011 - virus isolate A/Portugal/03/2011 (UE outlier), also showed HRI by the drug (≈250-fold higher IC_{50}; Figure 4.4D). The remaining 66 seasonal A(H1N1) and 146 A(H1N1)pdm09 viruses, as well as all 144 A(H3N2) and 142 influenza B viruses analysed, showed NI by OS (≤4-fold (A(H1N1)pdm09, A(H3N2)) or ≤2-fold (seasonal A(H1N1), influenza B) higher IC_{50}) (Figure 4.4A-D).

4.1.4.1.1.2 Zanamivir

All or nearly all (80.8% to 98.6%) influenza A viruses from the different subtypes and influenza seasons or 2009 pandemic period, exhibited an IC_{50} value within the normal range (Table 4.3). The only exception were the A(H1N1)pdm09 viruses from 2012/2013, in which only one (20.0%) of the viruses had an IC_{50} value within this range. Regarding influenza B viruses, all or nearly all (90.7%, 95.7%) viruses from 2005/2006, 2007/2008, and the two latter seasons (2011/2012 and 2012/2013) were non-outlier, while none or

only one (50.0%) of the viruses from the remaining influenza seasons and 2009 pandemic period had an IC_{50} value within the normal range. The only exception was in 2004/2005, with 70.8% of the viruses presenting a value within this range (Table 4.3). IC_{50} value ranges of non-outlier viruses are presented in Table 4.3.

Table 4.3 Summary of phenotypic zanamivir IC_{50} outlying results.

Influenza virus type/subtype	ZANAMIVIR										
	Influenza season / 2009 pandemic period [a]	Number of viruses tested	NON-OUTLIER VIRUSES		OUTLIER VIRUSES - Number (%)						***TOTAL***
			Number (%)	IC_{50} value range (nM)	UPPER			LOWER			
					Mild	Extreme	***TOTAL***	Mild	Extreme	TOTAL	
Former seasonal A(H1N1)	2004/2005	3	3 (100.0)	1.10 - 1.76	0 (0.0)	0 (0.0)	*0 (0.0)*	0 (0.0)	0 (0.0)	0 (0.0)	*0 (0.0)*
	2005/2006	27	27 (100.0)	0.52 - 3.97	0 (0.0)	0 (0.0)	*0 (0.0)*	0 (0.0)	0 (0.0)	0 (0.0)	*0 (0.0)*
	2006/2007	-[b]	-	-	-	-	-	-	-	-	-
	2007/2008	28	26 (92.9)	0.44 - 1.73	0 (0.0)	0 (0.0)	*0 (0.0)*	2 (7.1)	0 (0.0)	2 (7.1)	***2 (7.1)***
	2008/2009	18	17 (94.4)	0.67 - 1.90	0 (0.0)	0 (0.0)	*0 (0.0)*	1 (5.6)	0 (0.0)	1 (5.6)	***1 (5.6)***
	Pandemic period	0	-	-	-	-	-	-	-	-	-
	TOTAL	76	73 (96.1)	0.44 - 3.97	0 (0.0)	0 (0.0)	*0 (0.0)*	3 (3.9)	0 (0.0)	3 (3.9)	***3 (3.9)***
A(H3N2)	2004/2005	71	70 (98.6)	0.34 - 1.74	0 (0.0)	0 (0.0)	*0 (0.0)*	1 (1.4)	0 (0.0)	1 (1.4)	***1 (1.4)***
	2005/2006	1	1 (100.0)	0.73	0 (0.0)	0 (0.0)	*0 (0.0)*	0 (0.0)	0 (0.0)	0 (0.0)	*0 (0.0)*
	2006/2007	20	19 (95.0)	0.36 - 0.73	0 (0.0)	0 (0.0)	*0 (0.0)*	0 (0.0)	1 (5.0)	1 (5.0)	***1 (5.0)***
	2007/2008	-[b]	-	-	-	-	-	-	-	-	-
	2008/2009	20	19 (95.0)	0.32 - 0.58	0 (0.0)	0 (0.0)	*0 (0.0)*	1 (5.0)	0 (0.0)	1 (5.0)	***1 (5.0)***
	Pandemic period	0	-	-	-	-	-	-	-	-	-
	2010/2011	0	-	-	-	-	-	-	-	-	-
	2011/2012	26	24 (92.3)	0.68 - 1.52	1 (3.8)	1 (3.8)	***2 (7.7)***	0 (0.0)	0 (0.0)	0 (0.0)	***2 (7.7)***
	2012/2013	1	1 (100.0)	1.1	0 (0.0)	0 (0.0)	*0 (0.0)*	0 (0.0)	0 (0.0)	0 (0.0)	*0 (0.0)*
	TOTAL	139	134 (96.4)	0.32 - 1.74	1 (0.7)	1 (0.7)	***2 (1.4)***	2 (1.4)	1 (0.7)	3 (2.2)	***5 (3.6)***
B	2004/2005 (YAM)	24	17 (70.8)	5.62 - 9.72	2 (8.3)	0 (0.0)	***2 (8.3)***	2 (8.3)	3 (12.5)	5 (20.8)	***7 (29.2)***
	2005/2006 (VIC)	47	45 (95.7)	9.66 - 24.03	0 (0.0)	0 (0.0)	*0 (0.0)*	0 (0.0)	2 (4.3)	2 (4.3)	***2 (4.3)***
	2006/2007	2	1 (50.0)	3.93	1 (50.0)	0 (0.0)	***1 (50.0)***	0 (0.0)	0 (0.0)	0 (0.0)	***1 (50.0)***
	2007/2008 (YAM)	43	39 (90.7)	1.64 - 9.16	1 (2.3)	0 (0.0)	***1 (2.3)***	1 (2.3)	2 (4.7)	3 (7.0)	***4 (9.3)***
	2008/2009 (VIC)	1	0 (0.0)	-	1 (100.0)	0 (0.0)	***1 (100.0)***	0 (0.0)	0 (0.0)	0 (0.0)	***1 (100.0)***
	Pandemic period (VIC)	3	0 (0.0)	-	3 (100.0)	0 (0.0)	***3 (100.0)***	0 (0.0)	0 (0.0)	0 (0.0)	***3 (100.0)***
	2010/2011	2	0 (0.0)	-	2 (100.0)	0 (0.0)	***2 (100.0)***	0 (0.0)	0 (0.0)	0 (0.0)	***2 (100.0)***
	2011/2012 (YAM)	1	1 (100.0)	7.45	0 (0.0)	0 (0.0)	*0 (0.0)*	0 (0.0)	0 (0.0)	0 (0.0)	*0 (0.0)*
	2012/2013 (YAM)	6	6 (100.0)	5.71 - 8.72	0 (0.0)	0 (0.0)	*0 (0.0)*	0 (0.0)	0 (0.0)	0 (0.0)	*0 (0.0)*
	TOTAL	129	109 (84.5)	1.64 - 24.03	10 (7.8)	0 (0.0)	***10 (7.8)***	3 (2.3)	7 (5.4)	10 (7.8)	***20 (15.5)***
A(H1N1)pdm09	Pandemic period	116	110 (94.8)	0.40 - 0.99	4 (3.4)	0 (0.0)	***4 (3.4)***	2 (1.7)	0 (0.0)	2 (1.7)	***6 (5.2)***
	2010/2011	26	21 (80.8)	0.41 - 0.80	1 (3.8)	1 (3.8)	***2 (7.7)***	2 (7.7)	1 (3.8)	3 (11.5)	***5 (19.2)***
	2011/2012	-[b]	-	-	-	-	-	-	-	-	-
	2012/2013	5	1 (20.0)	0.81	0 (0.0)	4 (80.0)	***4 (80.0)***	0 (0.0)	0 (0.0)	0 (0.0)	***4 (80.0)***
	TOTAL	147	132 (89.8)	0.40 - 0.99	5 (3.4)	5 (3.4)	***10 (6.8)***	4 (2.7)	1 (0.7)	5 (3.4)	***15 (10.2)***

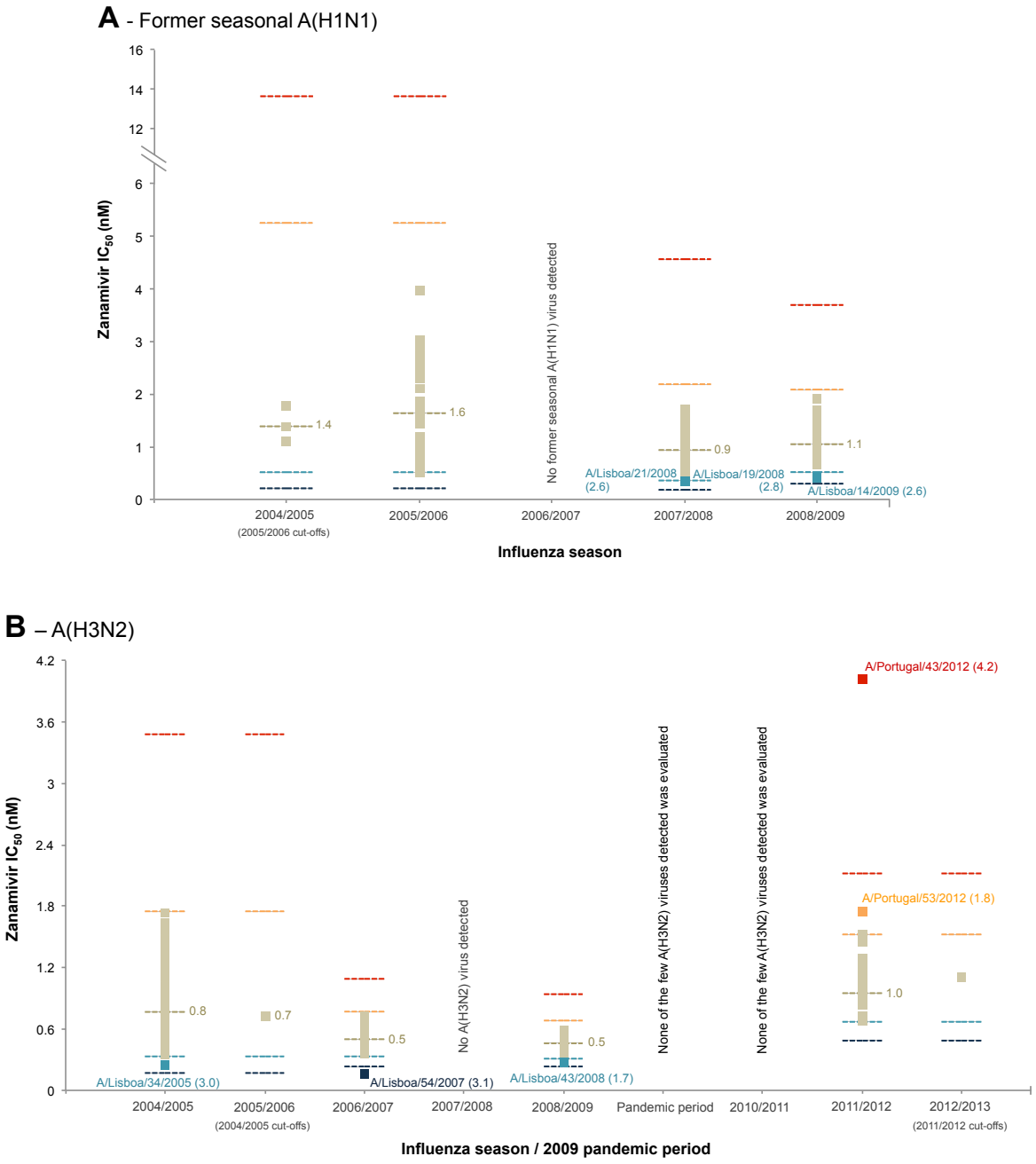
VIC: Victoria; YAM: Yamagata

[a] The predominant influenza B lineage is indicated within brackets after the designation of the time period (lineage differentiation based on the antigenic and/or genetic characteristics of the virus hemagglutinin); [b] No influenza virus was detected.

These results were determined and annotated as described in Table 4.2.

The lowest overall IC_{50} values exhibited by non-outlier influenza A viruses were similar across the different subtypes (0.32 to 0.44nM) (Table 4.3). The same similarity was not, however, observed at the upper limit of the overall range. Seasonal A(H1N1) non-outlier viruses yielded higher IC_{50} values that ranged up to 3.97nM, followed by A(H3N2) non-

outlier viruses that exhibited a maximum IC_{50} value of 1.74nM, and then by A(H1N1)pdm09 non-outlier viruses, which IC_{50} values extended up to 0.99nM. Influenza B non-outlier viruses exhibited far higher IC_{50} values distributed over a much wider range that overall varied from 1.64 to 24.03nM (Table 4.3). Notably, the highest values within this range were exhibited by the non-outlier viruses from 2005/2006, with all belonging to the B/VIC lineage (9.66 to 24.03nM). Influenza B non-outlier viruses from all other seasons were predominantly or exclusively from B/YAM lineage (Figure 4.5C).



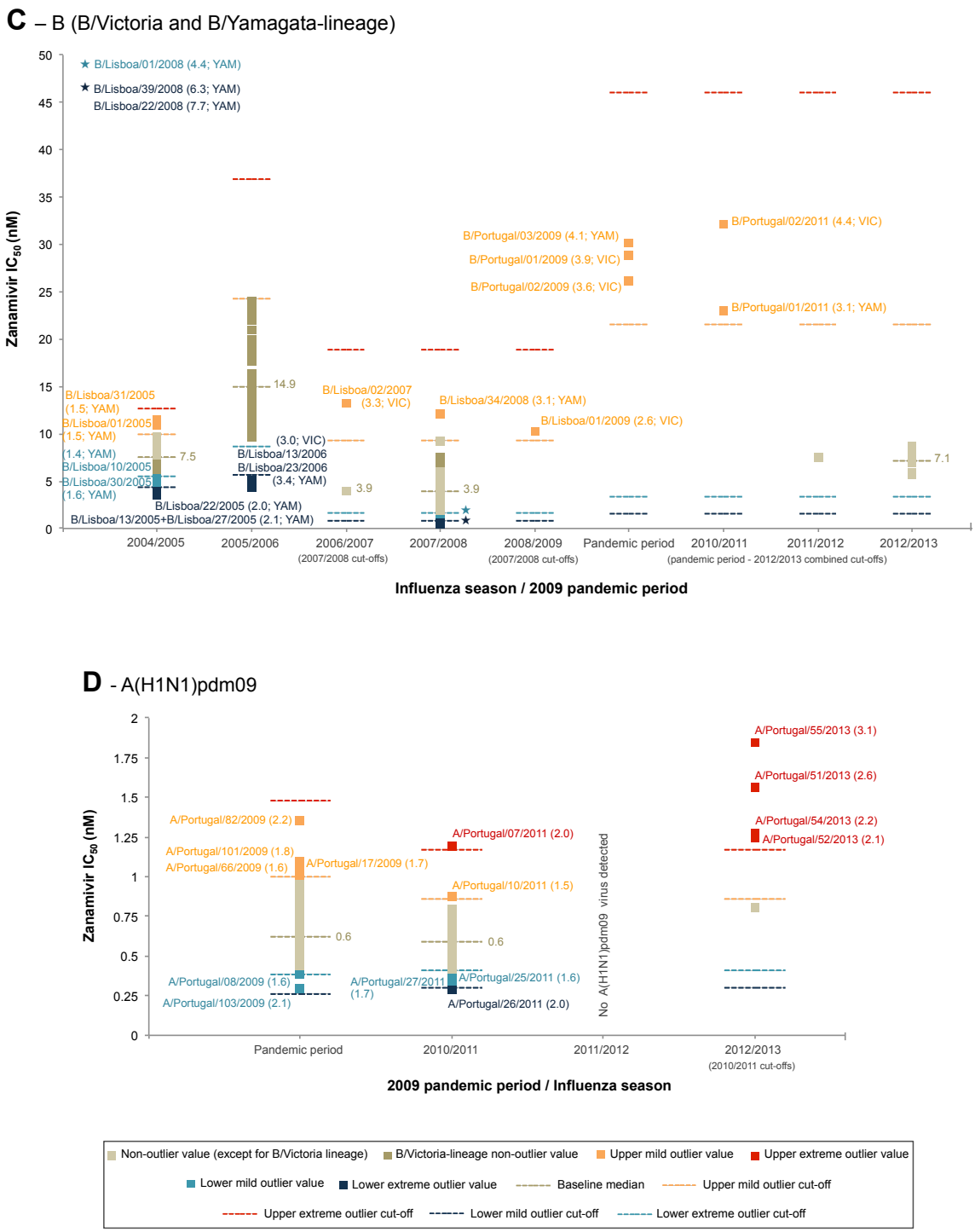


Figure 4.5 Zanamivir IC_{50} values of circulating former seasonal A(H1N1) (**A**), A(H3N2) (**B**), influenza B (**C**) and A(H1N1)pdm09 (**D**) viruses.

IC_{50} values, cut-offs and baseline median estimates were determined and/or annotated as described in Figure 4.4. All complementary annotations to IC_{50} plots are also described in this previous figure.

ZA IC_{50} outlier viruses belonged to virtually all 4 categories, except in seasonal A(H1N1) subtype, in which all three outlier viruses were LM (Table 4.3). Only few outlier viruses

were also detected in A(H3N2) subtype (n=5; 3.6%), with the number increasing considerably in both influenza type B (n=20; 15.5%) and A(H1N1)pdm09 subtype (n=15; 10.2%). Additional information on ZA IC_{50} outlier viruses is presented at Figure 4.5 A-D, in which are also indicated the IC_{50} cut-offs and baseline median.

The three seasonal A(H1N1) LM outlier viruses were from 2007/2008 (n=2; 7.1%) and 2008/2009 (n=1; 5.6%), presenting an ≈3-fold decrease in IC_{50}, compared to the corresponding baseline median (Table 4.3; Figure 4.5A).

Two A(H3N2) viruses from 2011/2012 were identified as upper outlier – virus isolates A/Portugal/43/2012 (UE outlier) and A/Portugal/53/2012 (UM outlier), exhibiting, respectively, a 4 and ≈2-fold higher ZA IC_{50} value than the baseline median (Figure 4.5B). An ≈2 to 3-fold decrease in IC_{50} relative to baseline median, was observed for the three A(H3N2) lower outlier viruses identified (2 LM and 1 LE).

Influenza B IC_{50} outlier viruses were detected in all influenza seasons except the two later ones (2011/2012 and 2012/2013), and in the 2009 pandemic period. The frequency at which outlier viruses were detected varied between 4.3% and 50.0% during the first 4 seasons, settling at 100.0% from 2008/2009 onwards (Table 4.3). Half (10/20) of the outlier viruses identified were UM outlier, with the fold increase in IC_{50} relative to baseline median increasing slightly over time. Specifically, from ≤2-fold in 2004/2005 to ≈3-fold between 2006/2007 and 2008/2009, and then to ≈4-fold in both pandemic period and 2010/2011 (Figure 4.5C). Only the virus isolate B/Portugal/01/2011 from 2010/2011 exhibited a 3-fold higher IC_{50} value. It is important to note that both UM outlier viruses from 2006/2007 (virus isolate B/Lisboa/02/2007) and 2008/2009 (virus isolate B/Lisboa/01/2009) belonged to the B/VIC lineage and were identified against statistical cut-offs based on essentially IC_{50} values from B/YAM-lineage viruses (2007/2008). If analysed against the cut-offs estimated for 2005/2006 that were exclusively based on B/VIC-lineage IC_{50} values, both virus isolates would lose their status of outlier. However, it was followed the same approach used in the analysis of OS IC_{50} data. The fold decrease in IC_{50} relative to baseline median observed for lower outlier viruses (3 LM and 7 LE) also increased slightly over time. It started at ≤2-fold in 2004/2005 and then increased to ≈3-fold in 2005/2006 and to a range between 4 and ≈8-fold in 2007/2008 (Figure 4.5C). All lower outlier viruses except one (virus isolate

B/Lisboa/13/2006) belonged to the B/YAM lineage. The tendency of B/VIC-lineage viruses for exhibiting higher non-outlier IC_{50} values, compared to B/YAM-lineage viruses, observed in OS susceptibility (see section 4.1.4.1.1.1) was not observed for ZA (higher values in 2007/2008, but not in 2004/2005) (Figure 4.5C).

The frequency of A(H1N1)pdm09 ZA IC_{50} outlier viruses increased over time. Specifically, from 5.2% (n=6) in the pandemic period to 19.2% (n=5) in 2010/2011, and then to 80.0% (n=4) in 2012/2013 (Table 4.3). However, as above-mentioned for OS, the high outlier frequency observed in this later season might be an artefact of having analysed the values against the IC_{50} cut-offs estimated for 2010/2011. Most A(H1N1)pdm09 outlier viruses were upper outlier (n=10; 66.7%), equitably distributed between UM and UE outlier categories (Table 4.3). An ≈2 to 3-fold increase in IC_{50} relative to baseline median was observed for all upper outlier viruses (Figure 4.5D). The 5 A(H1N1)pdm09 viruses identified as lower outlier (4 LM and 1 LE) presented an ≈2-fold decrease in IC_{50}.

According to the WHO AVWG IC_{50} fold-change criteria (detailed in section 4.1.1), all 76 seasonal A(H1N1), 139 A(H3N2), 129 influenza B, and 147 A(H1N1)pdm09 viruses showed NI by ZA (≤2-fold (seasonal and 2009 pandemic A(H1N1)) or ≤4-fold (A(H3N2), influenza B) higher IC_{50}) (Figure 4.5A-D).

4.1.4.1.2 Baseline level of phenotypic susceptibility – variation over time and overall estimates

The baseline level of phenotypic susceptibility is given by the IC_{50} baseline median (standard measure), representing the natural *in vitro* susceptibility of circulating viruses to the drug. In this study, an IC_{50} outlier median was also exceptionally used as a measure of baseline susceptibility, since all seasonal A(H1N1) viruses from 2008/2009 were outlier, exhibiting extremely high OS IC_{50} values. Figure 4.6 shows the IC_{50} baseline and outlier (seasonal A(H1N1) plot, OS) median estimates plotted against time. Single IC_{50} non-outlier values were also included to have a reference for the natural *in vitro* susceptibility of the viruses of that influenza season. IC_{50} data was plotted in reverse

order to evidence the inverse relationship between IC_{50} and drug susceptibility (the higher the IC_{50} is, the lower the susceptibility will be).

Overall, the baseline level of phenotypic susceptibility of circulating human influenza viruses varied over time (2004/2005 to 2012/2013), but with no particular trend and in essentially different ways to OS and ZA.

A small but statistically significant decrease in the natural susceptibility of circulating seasonal A(H1N1) viruses to OS was observed between 2005/2006 and 2007/2008 ($p=0.022$), preceding the marked (≈270-fold) and significant decrease in 2008/2009 (526.35nM, IC_{50} outlier median; $p=0.001$)(Figure 4.6). The natural susceptibility of circulating seasonal A(H1N1) viruses to ZA also changed significantly between 2005/2006 and 2007/2008 ($p=0.003$), increasing ≈2-fold between the two seasons.

The natural susceptibility of circulating A(H3N2) viruses to both OS and ZA decreased significantly between 2008/2009 and 2011/2012 (2-fold decrease; $p<0.005$) (Figure 4.6). The baseline level of susceptibility in this later season was also significantly lower (≈2-fold) compared to that before 2008/2009 ($p<0.005$), with exception of 2004/2005 for ZA ($p=0.155$). The single OS and ZA IC_{50} non-outlier values available for 2012/2013 were higher than the baseline median estimated for 2011/2012 (Figure 4.6). However, the values were within the OS or ZA IC_{50} non-outlier value range obtained for this season, as evidenced in previous Figures 4.4B (OS) and 4.5B (ZA), which suggested similar natural susceptibility to both drugs.

The influenza B viruses from the pandemic period exhibited a significantly lower (2 to 3-fold) natural susceptibility to OS, compared to the viruses from all influenza seasons for which the IC_{50} baseline median was estimated and possible to compare statistically ($p_{exact}<0.0005$, except with 2012/2013 ($p_{exact}=0.024$)) (Figure 4.6). However, this may be an artefact of the use of pandemic period – 2012/2013 combined cut-offs that, as referred above (see section 4.1.4.1.1.1), probably prevented the three considerably higher OS IC_{50} values of the pandemic period to be classified as outlier. The natural susceptibility of circulating influenza B viruses to OS had already decreased significantly after 2004/2005, as evidenced by the statistically significant differences between the IC_{50} baseline median of 2004/2005 and all following influenza seasons and the pandemic period ($p_{(exact)}<0.0005$, except with 2012/2013 ($p=0.001$)) (Figure 4.6). Only compared

to 2005/2006 this significant difference was not observed (p_{exact}=0.212), but this could be related with the very small IC_{50} sample size for this season (n=2). Regarding ZA, a significantly lower (2 to ≈4-fold) natural susceptibility to the drug was detected in 2005/2006, compared to all other influenza seasons for which the IC_{50} baseline median was estimated (p<0.0005, except with 2012/2013 (p=0.023)) (Figure 4.6). This may be related with the co-circulation of two distinct lineages of influenza B viruses, since all non-outlier ZA IC_{50} values in 2005/2006 were from B/VIC-lineage viruses; while those in all other influenza seasons were exclusively or predominantly from B/YAM-lineage viruses (see Figure 4.5C). In fact, when comparing the overall natural susceptibility of B/VIC and B/YAM-lineage viruses (data not shown), this revealed to be significantly lower for B/VIC-lineage viruses, not only to ZA (p<0.0005; VIC_{IC50}=14.35nM, YAM_{IC50}=4.82nM) but also, although less significantly, to OS (p=0.002; VIC_{IC50}=21.27nM, YAM_{IC50}=17.28nM).

The natural susceptibility of circulating A(H1N1)pdm09 viruses to OS decreased slightly but significantly between the pandemic period and 2010/2011 (p=0.001) (Figure 4.6). No significant variation was observed between these two time periods in the virus natural susceptibility to ZA (p=0.272). Also, although the single ZA IC_{50} non-outlier value available for 2012/2013 was higher than the baseline median estimated for the pandemic period and 2010/2011, it was within both IC_{50} non-outlier value ranges (see Figure 4.5D), suggesting a similar natural susceptibility to the drug.

The natural *in vitro* susceptibility of circulating viruses to OS and ZA differed in all influenza virus types and subtypes, as evidenced in Figure 4.6 by comparison of the corresponding IC_{50} data in side-by-side plots. This difference was observed individually in the different influenza seasons or pandemic period and also globally (total time period). Seasonal A(H1N1) (except those from 2005/2006), A(H1N1)pdm09 and, particularly, influenza B viruses exhibited a lower natural susceptibility to OS (respectively, ≤2-fold, ≤3-fold, and ≤5-fold lower), while the inverse was observed for A(H3N2) subtype (≤3-fold higher susceptibility). All observed differences possible to test statistically were found to be significant ($p_{(exact)}$<0.0005, except for A(H3N2) in 2006/2007 (p_{exact}=0.009), 2008/2009 (p=0.053) and 2011/2012 (p=0.001), and for influenza B in 2012/2013 (p_{exact}=0.031)). Only the seasonal A(H1N1) viruses from 2005/2006 exhibited similar natural susceptibility to OS and ZA, as confirmed by statistical comparison (p=0.946).

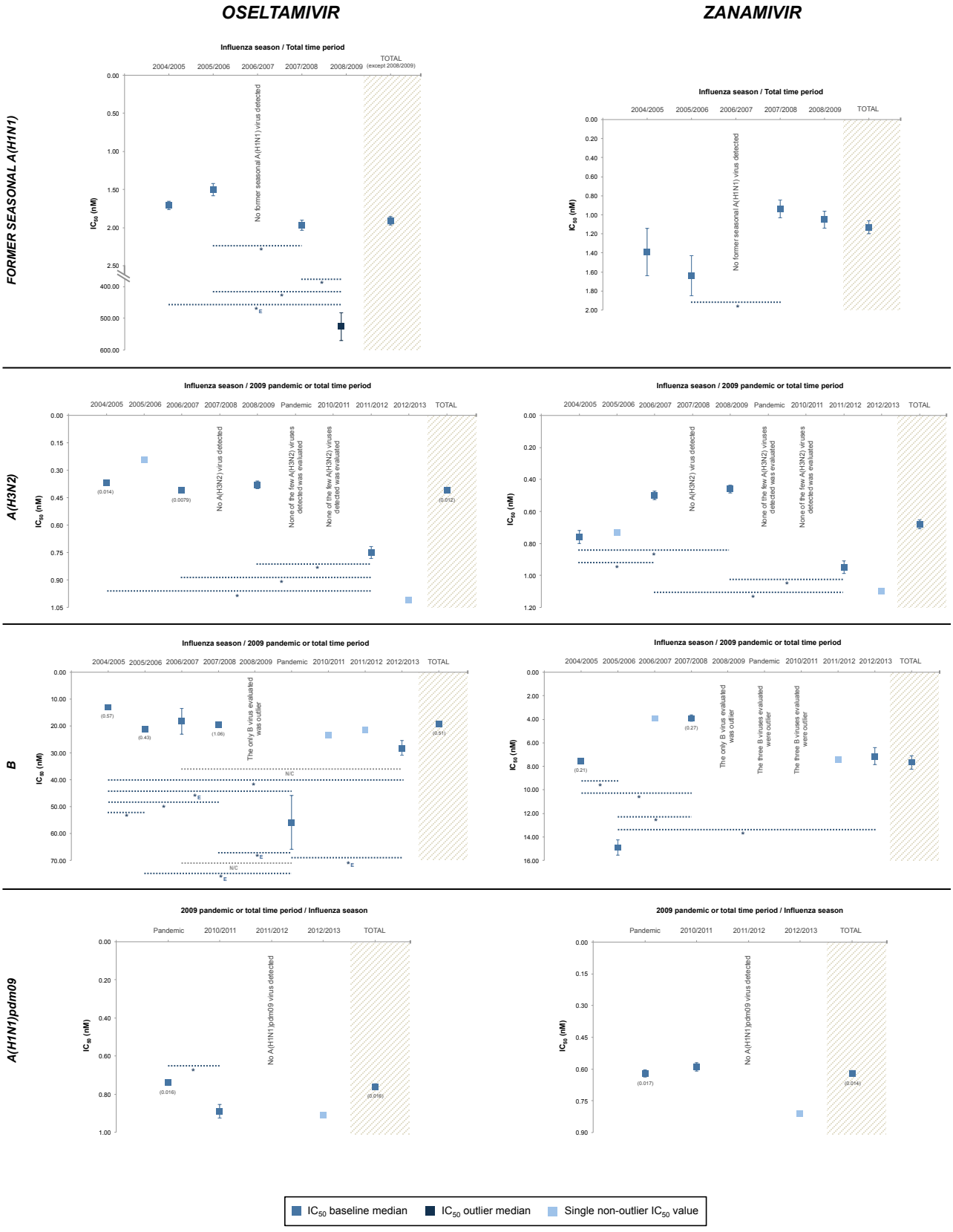


Figure 4.6 Baseline level of phenotypic susceptibility of circulating former seasonal A(H1N1), A(H3N2), influenza B and A(H1N1)pdm09 viruses, to oseltamivir (left) and zanamivir (right).

(Footnotes Figure 4.6)

The baseline level of phenotypic susceptibility is given by the IC_{50} baseline median (median without IC_{50} outlier values; standard measure). The oseltamivir (OS) IC_{50} outlier median of the former seasonal A(H1N1) viruses from 2008/2009 was exceptionally used as a measure of their baseline susceptibility, since all viruses tested were found to be upper extreme outlier to OS. Single IC_{50} non-outlier values are also indicated to have a reference for the baseline susceptibility in that influenza season. A colour coded-system composed by three different shades of blue was used to display these three different types of IC_{50} data (baseline median, outlier median, single value). IC_{50} data are plotted against time in reverse order due the inverse relationship between IC_{50} and phenotypic susceptibility (higher IC_{50} → lower phenotypic susceptibility). The vertical bars represent the standard error (SE) of the IC_{50} median estimates. When these vertical bars are not clearly visible (extremely small SE), the exact value is displayed in brackets below the corresponding IC_{50} median marker. A coloured background highlights the overall IC_{50} baseline median estimates. The existence of statistically significant differences among IC_{50} baseline or outlier median estimates was initially assessed by a Kruskal-Wallis H test and then, if confirmed, by post hoc pairwise comparisons performed using Dunn's (1964) procedure with a Bonferroni adjustment. A(H1N1)pdm09 IC_{50} baseline median estimates for the pandemic period and 2010/2011 were exceptionally compared by a Mann-Whitney U test. Also, multiple Mann-Whitney U tests were used in former seasonal A(H1N1) and influenza B type/subtype to calculate the exact statistical significance when comparing IC_{50} median estimates on which at least one was based on less than 5 values (n<5). No statistical inference could, however, be performed between the influenza B OS IC_{50} baseline median for 2006/2007 (n=2) and the pandemic period (n=3) or 2012/2013 (n=6), as both samples were too small to apply the test. The horizontal punctuated grey lines in the influenza B OS plot represent this lack of statistical comparison (N/C: Not compared). Both asymptotic (standard) and exact p-values were considered statistically significant at ≤0.05. The horizontal punctuated blue lines represent the statistically significant differences found, with the asterisk (*) and the asterisk followed by an E (*E) further indicating if the significant differences were based on, respectively, asymptotic or exact p-values.

Influenza virus type- and subtype-specific differences were also observed in the natural susceptibility of circulating viruses to OS and ZA. The only exception was observed for ZA, with A(H3N2) and A(H1N1)pdm09 viruses exhibiting a similar overall IC_{50} baseline median (0.68nM and 0.62nM, respectively) (Figure 4.6), confirmed by statistical comparison (p=0.178). The variation observed in the overall natural susceptibility of circulating viruses was similar to both NAIs and ordered as follows: influenza B **<** seasonal A(H1N1) **<** A(H1N1)pdm09 **<**$_{oseltamivir}$/**~**$_{zanamivir}$ A(H3N2). The variations observed individually in the different influenza seasons or pandemic period followed this overall ordering. Evidence for significant differences was found in all virus type or subtype comparisons possible to test statistically ($p_{(exact)}$<0.0005, except in 2006/2007 between influenza B and A(H3N2) (p_{exact}=0.017) for OS; and in 2004/2005 between seasonal A(H1N1) and A(H3N2) (p_{exact}=0.031) or influenza B (p_{exact}=0.002) for ZA).

4.1.4.2 Genotypic Testing

OS and/or ZA IC_{50} upper outlier viruses (UM and UE) were evaluated through NA and HA gene sequencing. The only exception were two A(H1N1)pdm09 upper outlier viruses

from 2012/2013 – virus isolates A/Portugal/51/2013 and A/Portugal/52/2013 (OS UM outlier; ZA UE outlier), for which was not available sufficient volume to perform genotypic testing. Also, only half (11/21) of the seasonal A(H1N1) OS UE outlier viruses from 2008/2009 were characterized regarding their HA sequence, and HA sequencing was unsuccessful for one influenza B ZA UM outlier virus from the pandemic period – virus isolate B/Portugal/01/2009.

More than 25% (limit set) of the lower outlier viruses detected within each influenza season or pandemic period for each influenza virus type or subtype, were evaluated through genotypic testing. Only the influenza B lower outlier viruses from 2004/2005 and 2005/2006 were not characterized regarding their NA and both NA and HA sequences, respectively.

4.1.4.2.1 Neuraminidase and hemagglutinin amino acid substitutions

4.1.4.2.1.1 Former seasonal influenza A(H1N1) viruses

The relevant NA and HA amino acid substitutions identified in seasonal A(H1N1) phenotypic outlier viruses are presented in Table 4.4, including their specific amino acid substitutions and further substitutions associated with NAI resistance.

NA H275Y amino acid substitution known to confer HRI by OS and peramivir (PER) *in vitro* and to cause clinical resistance to OS in influenza N1 NA viruses (see Table 1.2, Literature Review for detail), was identified in all 27 seasonal A(H1N1) UE outlier viruses showing HRI by OS (6 from 2007/2008; 21 from 2008/2009). These viruses also carried the NA D354G reversion amino acid substitution that, together with R222Q, V234M and D344N amino acid substitutions, these latter yielded by all seasonal A(H1N1) outlier and non-outlier viruses from 2007/2008 onwards, probably compensated for the fitness deficits of H275Y substitution (potential fitness-compensatory substitutions; detailed in section 1.5.2.2, Literature Review). NA D354G is considered a reversion substitution as was present in the seasonal A(H1N1) viruses circulating prior to 2007/2008. In 2007/2008, the circulating viruses acquired the NA G354D substitution that reverted to D354G in exclusively NA H275Y variant viruses. According to the 1918 N1 NA structure,

residue 354 is located at the NA surface, on the opposite side of the active site (Figure 4.7A).

Table 4.4 Former seasonal A(H1N1) phenotypic outlier virus neuraminidase and hemagglutinin specific amino acid substitutions and further substitutions associated with neuraminidase inhibitor resistance.

				FORMER SEASONAL A(H1N1)															
				NEURAMINIDASE										HEMAGGLUTININ					
														HA1[a]				HA2 (F Fusion sub-domain)	
	Influenza season	Virus isolate	Phenotypic information	TM domain	Stalk domain		Catalytic domain (globular head)							E' sub-domain	R sub-domain				
PHENOTYPIC UPPER OUTLIER	2005/2006	A/Lisboa/25/2006	OS UMo	None											None				
	2007/2008	A/Lisboa/08/2008	OS UMo	-	-	-	R222Q[c]	V234M[c]	D344N[c]	-	-	-	-	-	-			V201I	-
		A/Lisboa/02/2008	OS UEo, HRI	-	-	-	R222Q[c]	V234M[c]	***H275Y***	D344N[c]	***D354G***[d]	-	-	None					
		A/Lisboa/03/2008	OS UEo, HRI	-	-	-	R222Q[c]	V234M[c]	***H275Y***	D344N[c]	***D354G***[d]	-	-	None					
		A/Lisboa/11/2008	OS UEo, HRI	-	-	-	R222Q[c]	V234M[c]	***H275Y***	D344N[c]	***D354G***[d]	-	-	None					
		A/Lisboa/20/2008	OS UEo, HRI	-	-	-	R222Q[c]	V234M[c]	***H275Y***	D344N[c]	***D354G***[d]	-	-	M271I	-			-	
		A/Lisboa/27/2008	OS UEo, HRI	-	-	-	R222Q[c]	V234M[c]	***H275Y***	D344N[c]	***D354G***[d]	-	-	-	E194K			E165K	-
		A/Lisboa/28/2008	OS UEo, HRI	-	-	-	R222Q[c]	V234M[c]	E259K/E[e]	***H275Y***	D344N[c]	***D354G***[d]	-	-	D186G			N71D/N[e]	-
		A/Lisboa/29/2008	OS UEo, HRI	-	I54T	-	R222Q[c]	V234M[c]	***H275Y***	D344N[c]	***D354G***[d]	***T435R***	-	-	***G152E***	***A189T***[f]		-	
		A/Lisboa/39/2008	OS UEo, HRI	-	***G41R***	***I48M***	R222Q[c]	V234M[c]	***I241L***	***H275Y***	D344N[c]	***D354G***[d]	-	-	***A189T***[f]	-		-	
		A/Lisboa/40/2008	OS UEo, HRI	-	-	-	R222Q[c]	V234M[c]	***H275Y***	D344N[c]	***D354G***[d]	-	-	-	***A189T***[f]	E242G		-	
		A/Lisboa/41/2008	OS UEo, HRI	-	V75F	-	R222Q[c]	V234M[c]	***H275Y***	D344N[c]	***D354G***[d]	-	-	-	***A189T***[f]	-		-	
		A/Lisboa/2/2009	OS UEo, HRI	-	-	-	R222Q[c]	V234M[c]	***H275Y***	D344N[c]	***D354G***[d]	-	-	-	***A189T***[f]	-		-	
		A/Lisboa/3/2009	OS UEo, HRI	-	-	-	R222Q[c]	V234M[c]	***H275Y***	D344N[c]	***D354G***[d]	***T435R***	-	-	***G152E***	***A189T***[f]		-	
		A/Lisboa/5/2009	OS UEo, HRI	-	***G41R***	***I48M***	R222Q[c]	V234M[c]	***I241L***	***H275Y***	D344N[c]	***D354G***[d]	-	-	***A189T***[f]	-		-	
		A/Lisboa/6/2009	OS UEo, HRI	-	***G41R***	***I48M***	R222Q[c]	V234M[c]	***I241L***	***H275Y***	D344N[c]	***D354G***[d]	-			-[g]			
		A/Lisboa/7/2009	OS UEo, HRI	-	-	-	R222Q[c]	V234M[c]	***H275Y***	D344N[c]	***D354G***[d]	-	-	-	***A189T***[f]	-		-	
		A/Lisboa/13/2009	OS UEo, HRI	-	***G41R***	-	R222Q[c]	V234M[c]	***I241L***	***H275Y***	D344N[c]	***D354G***[d]	S364N			-[g]			
	2008/2009	A/Lisboa/14/2009[b]	OS UEo, HRI	-	***G41R***	-	R222Q[c]	V234M[c]	***I241L***	***H275Y***	D344N[c]	***D354G***[d]	-	-	***A189T***[f]	-		-	
		A/Lisboa/15/2009	OS UEo, HRI	-	***G41R***	-	R222Q[c]	V234M[c]	***I241L***	***H275Y***	D344N[c]	***D354G***[d]	-			-[g]			
		A/Lisboa/16/2009	OS UEo, HRI	-	***G41R***	***I48M***	R222Q[c]	V234M[c]	***I241L***	***H275Y***	D344N[c]	***D354G***[d]	-			-[g]			
		A/Lisboa/17/2009	OS UEo, HRI	-	***G41R***	***I48M***	R222Q[c]	V234M[c]	***I241L***	***H275Y***	D344N[c]	***D354G***[d]	-			-[g]			
		A/Lisboa/18/2009	OS UEo, HRI	-	-	-	R222Q[c]	V234M[c]	***H275Y***	D344N[c]	***D354G***[d]	-	-			-[g]			
		A/Lisboa/19/2009	OS UEo, HRI	-	***G41R***		R222Q[c]	V234M[c]	***I241L***	***H275Y***	D344N[c]	***D354G***[d]	-			-[g]			
		A/Lisboa/20/2009	OS UEo, HRI	-	***G41R***	***I48M***	R222Q[c]	V234M[c]	***I241L***	***H275Y***	D344N[c]	***D354G***[d]	-	E99G	***A189T***[f]	-		R75K	I91F
		A/Lisboa/21/2009	OS UEo, HRI	-	-	-	R222Q[c]	V234M[c]	***H275Y***	D344N[c]	***D354G***[d]	***T435A***	-			-[g]			
		A/Lisboa/23/2009	OS UEo, HRI	-	-	-	R222Q[c]	V234M[c]	***H275Y***	D344N[c]	***D354G***[d]	***T435A***	-			-[g]			
		A/Lisboa/24/2009	OS UEo, HRI	-	***G41R***		S105N	R222Q[c]	V234M[c]	***I241L***	***H275Y***	D344N[c]	***D354G***[d]	-	***A189T***[f]	-		-	
		A/Lisboa/25/2009	OS UEo, HRI	-	-	-	R222Q[c]	V234M[c]	***H275Y***	D344N[c]	***D354G***[d]	-	-			-[g]			
PHENOTYPIC LOWER OUTLIER	2005/2006	A/Lisboa/24/2006	OS LMo	None										-	I116M			-	
	2007/2008	A/Lisboa/77/2007	OS LMo	M23T	-	-	R222Q[c]	V234M[c]	D344N[c]	-	-	-	-			None			
		A/Lisboa/09/2008	OS LMo	-	-	-	R222Q[c]	V234M[c]	D344N[c]	-	-	-	-			-[g]			
		A/Lisboa/10/2008	OS LMo	-	-	-	R222Q[c]	V234M[c]	D344N[c]	-	-	-	-			-[g]			
		A/Lisboa/14/2008	OS LEo	-	-	-	R222Q[c]	V234M[c]	D344N[c]	-	-	-	-			None			
		A/Lisboa/19/2008	ZA LMo	-	-	-	R222Q[c]	V234M[c]	D344N[c]	-	-	-	-			-[g]			

E': Vestigial esterase; HRI: Highly reduced inhibition; LEo: Lower extreme outlier; LMo: Lower mild outlier; OS: Oseltamivir; R: Receptor binding; TM: Transmembrane; UEo: Upper extreme outlier; UMo: Upper mild outlier; ZA: Zanamivir

Colour legend:

Associated with HRI by OS and peramivir *in vitro* and know to cause clinical resistance to OS - influenza N1 neuraminidase (NA) viruses

Potential compensatory substitution of the fitness deficits of NA H275Y substitution in former seasonal A(H1N1) viruses

[a] Position numbering without the N-terminal signal sequence (amino acid residues 1-17); [b] LMo against ZA; [c] Identified in all former seasonal A(H1N1) viruses from 2007/2008 onwards tested (outlier and non-outlier) and in virtually all reference viruses from 2005/2006 (V234M) or 2006/2007 (R222Q and D344N) onwards - exceptionally identified against the NA sequences of the viruses circulating before that (consensus sequence with 222Q, 234M and 344N); [d] Reversion substitution characteristic of the NA H275Y variant viruses circulating since 2007/2008 - exceptionally identified against the NA sequences of the H275 wild-type viruses from 2007/2008 (consensus sequence with 354G); [e] Mixed virus population; [f] Shared by all NA H275Y variant viruses from 2008/2009 (tested + reference); also detected in few previously circulating H275 wild-type and H275Y mutant reference viruses (from 2006 to 2008); [g] Not sequenced.

(Footnotes Table 4.4 cont.)
NA and hemagglutinin (HA) nucleotide sequences of tested (n=57 NA; n=40 HA) and reference (n=22 NA; n=23 HA) former seasonal A(H1N1) viruses were aligned by Clustal W method, edited and translated into the amino acid sequence in MEGA5. The accession number of the nucleotide sequences of the viruses tested shared through public-access databases can be found in Supplementary data (Table S4.1), as well as detailed information on the nucleotide sequences of the worldwide reference viruses collected for this study (Table S4.2). NA and HA amino acid substitutions were identified against, respectively, the seasonal N1 and H1 consensus sequences, with exception of the few substitutions described above (see footnotes c and d). The amino acid substitutions are indicated using the subtype specific numbering scheme (seasonal N1 and H1 numbering) and are displayed in order and by the structural domain (NA) or sub-domain (HA) of the protein on which occurred. The location of the NA structural domains in the seasonal N1 monomer was based on Colman [8] and Wei [9], while the location of the HA structural sub-domains in the seasonal H1 monomer was established according to Rosenthal *et al.* [10] and Gamblin *et al.* [11]. The amino acid substitutions shared by two or more UEo viruses are highlighted in bold and italic, while those occurring at residues belonging to the HA receptor binding site (based on Gamblin *et al.* [11]) are underlined. Detailed information on the NA amino acid substitutions associated with NA inhibitor resistance can be found at Table 1.2 (H275Y) or section 1.5.2.2 (potential fitness-compensatory mutations) of Literature Review.

Four different combinations of specific amino acid substitutions were identified in the NA of the seasonal A(H1N1) HRI viruses from 2008/2009: (1) H275Y + D354G (combination carried by those from 2007/2008); (2) H275Y + D354G + T435R; (3) H275Y + D354G + G41R + I241L; and (4) H275Y + D354G + G41R + I48M + I241L. These different combinations were not, however, associated with different fold increases in OS IC_{50}. Also, the amino acid substitutions distinguishing the combinations occurred at residues located at either some distance (I241L) or far away (T435R, G41R, and I48M) from the NA active site and residue 275. Residues 241 and 435 are, respectively, buried and at the surface of the NA globular head (Figure 4.7A), while residues 41 and 48 belong to the stalk domain of the protein (Table 4.4). Further NA specific amino acid substitutions identified in single HRI viruses from either 2007/2008 or 2008/2009 also lie at the surface of the globular head (E259K/E, S364N; Figure 4.7A) or within the stalk domain (I54T, V75F; Table 4.4).

Seasonal A(H1N1) UM and lower (LM and LE) outlier viruses carried no specific NA amino acid substitution, with exception of the virus isolate A/Lisboa/77/2007 (LM outlier) that contained the NA M23T substitution in the transmembrane domain (Table 4.4).

Regarding HA amino acid substitutions, all seasonal A(H1N1) HRI viruses from 2008/2009 evaluated through HA sequencing (n=11) carried the HA A189T amino acid substitution, with two of them – virus isolates A/Lisboa/29/2008 and

A/Lisboa/03/2009, further carrying HA G152E amino acid substitution (Table 4.4). Residues 189 and 152 are located at or near the 190-α-helix of the HA receptor binding site (RBS) (Figure 4.7B) and within antigenic site Sb [12,13]. All other HA amino acid substitutions identified in single HRI viruses from 2008/2009 – virus isolates A/Lisboa/40/2008 (E242G) and A/Lisboa/20/2009 (E99G, R75K (HA2), and I91F (HA2)), occurred away from the RBS. But, two of them - E99G and R75K (HA2), are located at or in the vicinity of the putative second sialic acid binding site on HA (Figure 4.7B). Two seasonal A(H1N1) HRI viruses from 2007/2008 – virus isolates A/Lisboa/27/2008 and A/Lisboa/28/2008, carried an amino acid substitution at the 190-α-helix of the HA RBS - E194K and D186G, respectively (Figure 4.7B), with residue 186 being directly involved in receptor binding. Both residues 194 and 186 belong to the antigenic site Sb. Virus isolate A/Lisboa/28/2008 contained a second specific amino acid substitution located at the putative second sialic acid HA binding site - N71D/N (Figure 4.7B). All other 4 HRI viruses from 2007/2008, as well as the remaining seasonal A(H1N1) outlier viruses evaluated, contained either no specific HA amino acid substitution (majority) or a single HA amino acid substitution located away from the RBS (I116M, M271I, V201I (HA2)) (Table 4.4; Figure 4.7B).

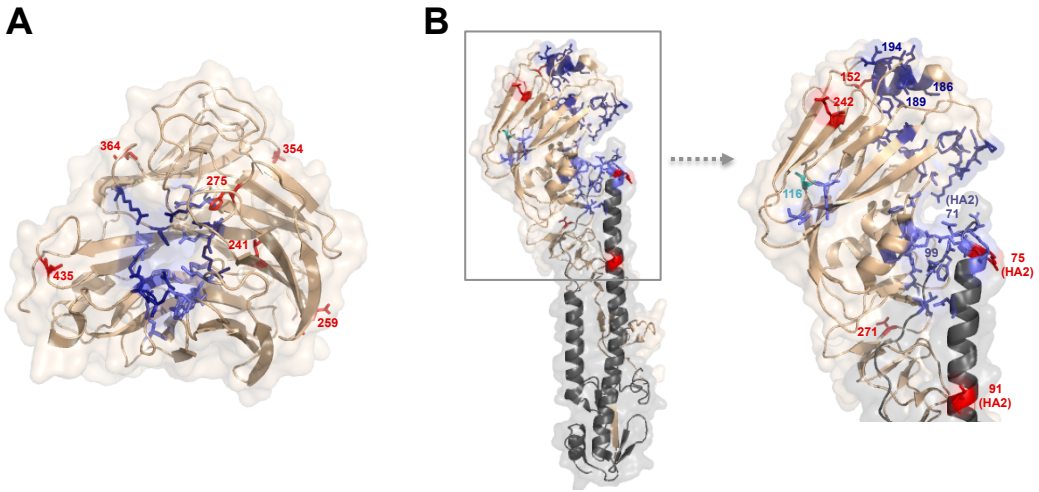


Figure 4.7 Mapping of the neuraminidase **(A)** and hemagglutinin **(B)** residues on which were detected the amino acid substitutions specific of former seasonal A(H1N1) phenotypic outlier viruses and/or associated with neuraminidase inhibitor resistance, onto the three-dimensional structure of the protein.

The figures were generated in PyMOL, using the protein structure file previously retrieved from the RCSB Protein Data Bank. **Panel A**: structure of the globular head of the neuraminidase (NA) from the former seasonal A(H1N1) virus A/Brevig Mission/01/1918 (PDB ID: 3B3Q; only the globular head of one of the two monomers described in the file is shown).

(Footnotes Figure 4.7 cont.)
The residues forming the NA active site (based on Colman *et al.* [14]) are coloured in dark blue (functional residues) or light purple (framework residues) and shown as sticks. **Panel B**: structure of the hemagglutinin (HA) from the former seasonal A(H1N1) virus A/South Carolina/01/1918 (PDB ID: 1RUZ; only one monomer of the HA trimer described in the file is shown). The HA1 subunit is displayed in light golden and the HA2 subunit in dark grey. The receptor binding site (RBS) residues (based on Gamblin *et al.* [11]) are shown as dark blue sticks, while the residues forming the putative second sialic acid binding site on HA (based on Sauter *et al.* [15]) are displayed as light purple sticks. **Panel A and B**: the residues on which were detected the amino acid substitutions are shown as sticks that are colour-coded according to the phenotypic outlier category of the virus(es) carrying the substitution: red - upper extreme outlier; orange - upper mild outlier; turquoise blue - lower mild outlier. The residues belonging to the HA RBS or second binding site maintained, however, the colour assigned to the functional site. NA residues located outside the globular head (residues 23, 41, 48, 54 and 75) could not be displayed (panel A). HA2 residue 201 was also missing in the structure file used (HA2 residues 1 to 160) and thereby is not displayed (panel B).

4.1.4.2.1.2 Influenza A(H3N2) viruses

NA I222V amino acid substitution known to confer a 2 or 4-fold decreased susceptibility to OS in A(H3N2) viruses (see Table 1.2, Literature Review for detail) was identified in an UE outlier virus from 2011/2012 with an ≈4-fold increase in both OS and ZA IC_{50} - virus isolate A/Portugal/43/2012 (Table 4.5). The effect of this substitution on ZA susceptibility is still unknown for A(H3N2) subtype but an ≈2-fold increase in IC_{50} has been reported for all other human influenza virus types and subtypes (detailed in Table 1.2, Literature Review). Besides NA I222V, the virus also carried NA D251G and S331N amino acid substitutions located, respectively, at some distance and far away from the active site (Figure 4.8A). All other A(H3N2) upper outlier viruses contained either no specific NA amino acid substitution or a single NA amino acid substitution located away from the active site (F42L, T138I/T) (Table 4.5; Figure 4.8A).

All A(H3N2) lower outlier viruses evaluated through NA sequencing contained also no specific amino acid substitution in their NA (Table 4.5). The NA D151N amino acid substitution identified as a mixed virus population in a LE outlier virus from 2006/2007 – virus isolate A/Lisboa/08/2007, is exceptionally indicated in Table 4.5 as several other substitutions at this same residue (D151A/E/G/V) are associated with (H)RI by NAI drugs (see Table 1.2, Literature Review for detail). Also, in influenza B viruses, this substitution is known to confer RI by PER. NA D151N substitution was detected in 11 reference A(H3N2) viruses from 2007 to 2012.

The non-outlier virus isolate A/Portugal/56/2013, recovered from a patient undergoing OS therapy, carried NA I262V and D399E amino acid substitutions located at the surface of the NA globular head (Table 4.5; Figure 4.8A). The clinical specimen yielding this virus isolate contained both amino acid substitutions (identical NA genetic background).

Table 4.5 Neuraminidase and hemagglutinin amino acid substitutions specific of the influenza A(H3N2) viruses classified as phenotypic outlier or recovered from a patient under antiviral therapy.

	Influenza season	Virus isolate	Phenotypic and other relevant information	A(H3N2)					
				NEURAMINIDASE				HEMAGGLUTININ HA1[a]	
				Stalk domain	Catalytic domain (globular head)			E´ sub-domain	R sub-domain
PHENOTYPIC UPPER OUTLIER	2006/2007	A/Lisboa/01/2007	OS UMo	None				None	
		A/Lisboa/54/2007[b]	OS Ueo	None				None	
	2011/2012	A/Portugal/29/2012	OS UMo	F42L	-			-	T135A
		A/Portugal/43/2012	OS/ZA UEo	-	I222V	D251G	S331N	None	
		A/Portugal/53/2012	ZA UMo	-	T138I/T[c]	-	-	None	
PHENOTYPIC LOWER OUTLIER	2004/2005	A/Lisboa/34/2005	ZA LMo	None				S54G	-
	2006/2007	A/Lisboa/08/2007	OS LEo	-	D151N/D[c,d]	-	-	None	
		A/Lisboa/18/2007	OS LMo	None				-[e]	
		A/Lisboa/20/2007	OS LEo	None				-[e]	
		A/Lisboa/24/2007	OS LMo	None				-[e]	
	2008/2009	A/Lisboa/43/2008	ZA LMo	None				None	
	2011/2012	A/Portugal/50/2012	OS LMo	None				None	
ASSOCIATED WITH ANTIVIRAL USE	2012/2013	A/Portugal/56/2013	Third-passage virus isolate	-	I262V	D399E	-	-	R261L
		Matching clinical specimen	Collected on day 2 of OS therapy	-	I262V	D399E	-	-	R261L

E': Vestigial esterase; LEo: Lower extreme outlier; LMo: Lower mild outlier; OS: Oseltamivir; R: Receptor binding; UEo: Upper extreme outlier; UMo: Upper mild outlier; ZA: Zanamivir

Colour legend: Associated with a normal inhibition ≥2-fold by OS *in vitro*, confirmed by reverse genetics – influenza A(H3N2) viruses

[a] Position numbering without the N-terminal signal sequence (amino acid residues 1-16); [b] LEo against ZA; [c] Mixed virus population; [d] Also identified in 11 reference A(H3N2) viruses from 2007 to 2012; exceptionally indicated as it occurs in a residue widely associated with (highly) reduced inhibition by neuraminidase (NA) inhibitors *in vitro* (D151A/E/G/V amino acid substitutions); [e] Not sequenced.

The amino acid substitutions were identified and are displayed as described in Table 4.4 (N2 and H3 numbering). The analysis involved a total of 124 NA and hemagglutinin (HA) sequences from tested (n=60 NA; n=57 HA (+1 clinical specimen)) and reference (n=63 NA; n=66 HA) A(H3N2) viruses (detailed information in Tables S4.1 and S4.2, Supplementary data). The location of the NA structural domains in the A(H3N2) N2 monomer was based on Colman [8], Colman and Ward [16], and Air and Laver [17]; while the location of the HA1 structural sub-domains in the A(H3N2) H3 monomer was established according to Ha *et al.* [18]. All substitutions occurring at residues belonging to the NA active site (based on Colman *et al.* [14]) or the HA receptor binding site (based on Russell *et al.* [19]) are underlined. Detailed information on the effect of NA I222V and D151A/E/G/V amino acid substitutions on A(H3N2) virus susceptibility can be found at Table 1.2, Literature Review.

HA T135A amino acid substitution was identified in an A(H3N2) UM outlier virus from 2011/2012 – virus isolate A/Portugal/29/2012 (Table 4.5). Residue 135 belongs to the HA RBS and is directly involved in sialic acid receptor binding. It also belongs to the

antigenic site A [20], with the threonine (T) to alanine (A) substitution resulting in the loss of a potential N-linked glycosylation site at position 133 (N133). All other A(H3N2) upper outlier viruses, as well as all lower outlier viruses evaluated through HA sequencing except one (virus isolate A/Lisboa/34/2005), carried no specific amino acid substitution. A/Lisboa/34/2005 carried HA S54G amino acid substitution located far away from the RBS (Figure 4.8B) and within antigenic site C [20]. The single A(H3N2) virus with known association to antiviral therapy (virus isolate A/Portugal/56/2013) contained HA R261L amino acid substitution located away from the RBS (Figure 4.8B) and within antigenic site E [20]. This virus also contained, although not specifically (also identified in 8 reference viruses from 2006 to 2012/2013), the HA R142G amino acid substitution that is known to occur clinically under OS drug pressure (detailed in section 1.5.2.1.2, Literature Review). The presence of both substitutions was confirmed in the matching clinical specimen. The HA S262N amino acid substitution know to emerge during antiviral therapy was identified in an A(H3N2) non-outlier virus from 2004/2005 with no known association with drug use (no information available) – virus isolate A/Lisboa/90/2005. The impact of both HA R142G and S262N substitutions on A(H3N2) virus NAI susceptibility is still unknown. But, according to the H3 HA structure of A/Victoria/361/2011 vaccine virus, these substitutions occur at either some distance (R142G) or far away (S262N) from the RBS (Figure 4.8B).

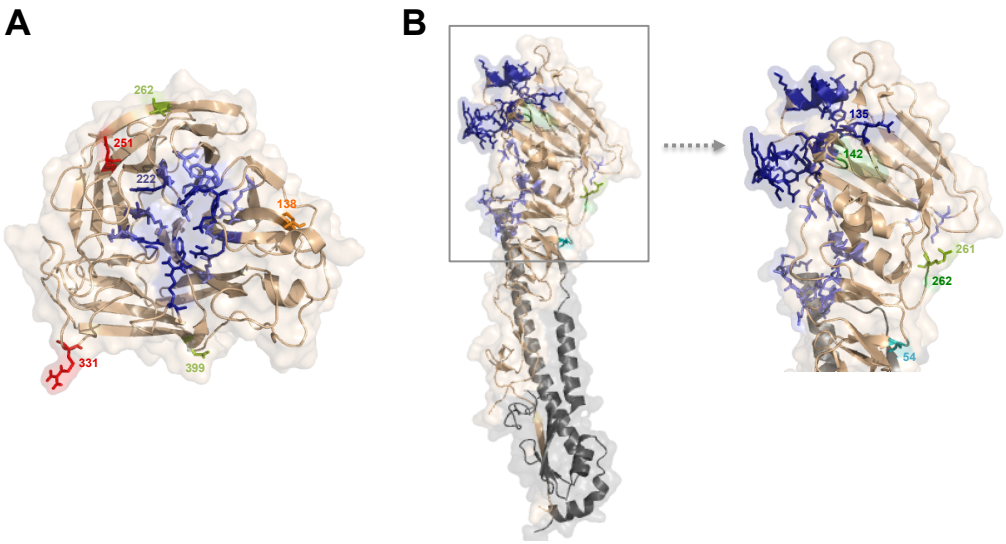


Figure 4.8 Mapping of the neuraminidase **(A)** and hemagglutinin **(B)** residues on which were detected the amino acid substitutions specific of the A(H3N2) viruses classified as phenotypic outlier or recovered from a patient under antiviral therapy, onto the three-dimensional structure of the protein.

(Footnotes Figure 4.8)

The figure was generated and annotated as described in Figure 4.7, using the structure of the neuraminidase (NA) globular head from the A(H2N2) virus A/Tokyo/3/1967 (PDB ID: 1NN2) (**panel A**) and the structure of the hemagglutinin (HA) from the A(H3N2) vaccine virus A/Victoria/361/2011 (PDB ID: 4WE8) (**panel B**). The residues defined as belonging to the receptor binding site (RBS) of H3 HA are based on Russell *et al.* [19]. Similarly to HA RBS residues, NA active site residues are not coloured according to the phenotypic outlier category of the virus carrying the substitution, maintaining the colour assigned to the corresponding type of active site residue (functional or framework). The additional residues on which occurred the amino acid substitutions specific of the influenza virus recovered from a patient undergoing antiviral therapy are also shown as sticks and are coloured in light green. **Panel B**: the HA residues on which were detected amino acid substitutions known to emerge during antiviral therapy are indicated using dark green sticks.

4.1.4.2.1.3 Influenza B viruses

NA D197N substitution known to confer RI by NAIs (up to 10-fold for OS and ZA; see Table 1.2, Literature Review for detail) was identified in a significant proportion (44.1%; pyrosequencing data not shown) of the virus population comprising the influenza B virus isolate B/Lisboa/34/2008 that exhibited an ≈3-fold increase in ZA IC_{50} (UM outlier) (Table 4.6).

Table 4.6 Neuraminidase and hemagglutinin amino acid substitutions specific of influenza B phenotypic outlier viruses.

	Influenza season / 2009 pandemic period	Virus isolate	Phenotypic information; B lineage	**INFLUENZA B** (B/VIC and B/YAM-lineage viruses)					
				NEURAMINIDASE			**HEMAGGLUTININ HA1**[a]		
				Stalk domain	Catalytic domain (globular head)		F' Fusion sub-domain	E' sub-domain	R sub-domain
PHENOTYPIC UPPER OUTLIER	2004/2005	B/Lisboa/01/2005	ZA UMo; YAM		None			None	
		B/Lisboa/12/2005	OS UMo; YAM	-	I240V[b]	E404G	T311A	K45R	-
		B/Lisboa/31/2005	ZA UMo; YAM		None			None	
	2005/2006	B/Lisboa/05/2006	OS UMo; VIC		None			None	
		B/Lisboa/11/2006	OS UMo; VIC		None			None	
	2006/2007	B/Lisboa/02/2007	ZA UMo; VIC	V63M	K125S	-		None	
	2007/2008	B/Lisboa/34/2008	ZA UMo; YAM	-	D197N/D[c]	-		None	
	2008/2009	B/Lisboa/01/2009	OS/ZA UMo; VIC	K65L	-			None	
	Pandemic period	B/Portugal/01/2009	ZA UMo; VIC	-	I287V	-		-[d]	
		B/Portugal/02/2009	ZA UMo; VIC	-	T311A	-	-	-	A146T
		B/Portugal/03/2009	ZA UMo; YAM		None			None	
	2010/2011	B/Portugal/01/2011	ZA UMo; YAM		None			None	
		B/Portugal/02/2011	OS/ZA UMo; VIC	-	V71G	-		None	
PHENOTYPIC LOWER OUTLIER	2004/2005	B/Lisboa/03/2005	OS LMo; YAM		-[d]			None	
		B/Lisboa/13/2005	ZA LEo; YAM		-[d]			None	
		B/Lisboa/22/2005	ZA LEo; YAM		-[d]			None	
	2007/2008	B/Lisboa/01/2008	ZA LMo; YAM		None			None	

E': Vestigial esterase; LEo: Lower extreme outlier; LMo: Lower mild outlier; OS: Oseltamivir; R: Receptor binding; UMo: Upper mild outlier; VIC: Victoria; YAM: Yamagata; ZA: Zanamivir

Colour legend: Associated with reduced inhibition by neuraminidase (NA) inhibitors (NAIs) *in vitro* - Influenza B viruses

[a] Position numbering without the N-terminal signal sequence (amino acid residues 1-15); [b] Also identified in an reference influenza B virus from 2009 - B/Uganda/U514/2009 (no information available regarding its susceptibility to NAIs); [c] Mixed virus population; [d] Not sequenced.

(Footnotes Table 4.6 cont.)

The amino acid substitutions were identified and are displayed as described in Table 4.4 (B/Victoria-lineage NA and HA numbering). The analysis involved a total of 94 NA and 108 hemagglutinin (HA) sequences from tested (n=38 NA; n=49 HA) and reference (n=56 NA; n=59 HA) influenza B viruses (detailed information in Tables S4.1 and S4.2, Supplementary data). The location of the NA structural domains in the influenza B NA monomer was based on Colman and Ward [16], and Flandorfer *et al.* [21]; while the location of the HA1 structural sub-domains in the influenza B HA monomer was established according to Wang *et al.* [22] and Ni *et al.* [23]. The amino acid substitutions occurring at residues belonging to the NA active site (based on Colman *et al.* [14]) are underlined. Detailed information on the effect of NA D197N amino acid substitution on influenza B virus susceptibility can be found at Table 1.2, Literature Review.

Further evaluation of virus susceptibility to OS by chemiluminescent assay did not confirm the non-outlier status of this mixed virus population of wild-type and mutant D197N viruses, but rather identify the virus isolate as UM outlier (≈2-fold increase in OS IC_{50}; data not shown). NA I240V amino acid substitution located closely to the active site (Figure 4.9A) was identified in the UM outlier virus isolate B/Lisboa/12/2005 and in a reference influenza B virus from 2009 with no available information regarding its susceptibility to NAIs (Table 4.6). All further NA amino acid substitutions specific of influenza B UM outlier viruses, except V63M and K65L (stalk), were located in the globular head catalytic domain (Table 4.6), but far away from the active site (Figure 4.9A). Approximately half (6/13) of the UM outlier viruses contained no specific amino acid substitution in NA. This was also the case of the single influenza B lower outlier virus evaluated through NA sequencing (Table 4.6).

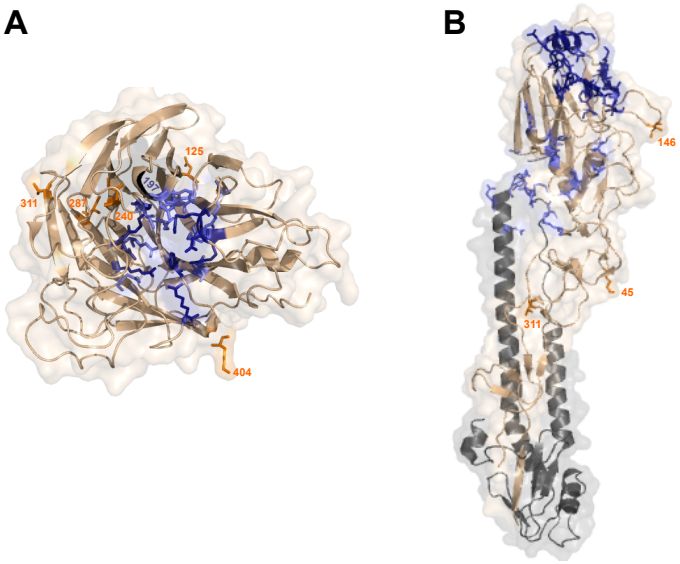


Figure 4.9 Mapping of the neuraminidase **(A)** and hemagglutinin **(B)** residues on which were detected the amino acid substitutions specific of the influenza B phenotypic outlier viruses, onto the three-dimensional structure of the protein.

(Footnotes Figure 4.9)
The figure was generated and annotated as described in Figure 4.7, using the structure of the neuraminidase (NA) globular head (PDB ID: 4CPL, only one globular head of the two described in the file is shown) (**panel A**) and of the hemagglutinin (HA) (PDB ID: 4FQM, only one monomer of the HA trimer described in the file is shown) (**panel B**) from the B/Victoria-lineage vaccine virus B/Brisbane/60/2008. The HA residues forming the influenza B RBS were defined according to Wang *et al.* [24] and Wang [25]. NA active site residues are not coloured according to the phenotypic outlier category of the virus carrying the substitution, maintaining the colour assigned to the corresponding type of active site residue (functional or framework). NA residue 71 could not be indicated as is missing in the structure file used (include residues 77 to 466).

All influenza B phenotypic outlier viruses evaluated through HA sequencing contained no specific amino acid substitution, with exception of the UM outlier virus isolates B/Lisboa/12/2005 (2004/2005) and B/Portugal/02/2009 (pandemic period). These contained, respectively, the HA K45R and T311A and HA A146T amino acid substitutions, located far away from the RBS (Table 4.6; Figure 4.9B).

4.1.4.2.1.4 2009 pandemic influenza A(H1N1) viruses

NA H275Y amino acid substitution was identified in the single A(H1N1)pdm09 UE outlier virus showing HRI by OS – virus isolate A/Portugal/03/2011 (Table 4.7). As above-mentioned, NA H275Y substitution is known to confer HRI by OS and PER *in vitro* and to cause clinical resistance to OS, in N1 NA influenza viruses. The clinical specimen yielding this virus isolate also carried the NA H275Y substitution, as detected in the initial H275Y screening performed at HCC on clinical specimens collected from patients suspected of having developed drug-resistant influenza during antiviral therapy (suspected case 3; see Table 3.2, Material and Methods). The two NA amino acid substitutions that may potentially compensate for the fitness deficits of H275Y substitution in A(H1N1)pdm09 viruses – V241I and N369K substitutions (detailed in section 1.5.2.2, Literature Review), were identified in this NA H275Y variant virus and in all other A(H1N1)pdm09 viruses from 2010/2011 onwards evaluated through NA sequencing (Table 4.7). A proportion of the virus population comprising the isolate A/Portugal/03/2011 also carried the NA P337S amino acid substitution (P337S/P mixed virus population; Table 4.7), located at some distance from either the residue 275 or the active site (Figure 4.10A).

The two A(H1N1)pdm09 upper outlier viruses from the pandemic period sharing an ≈4-fold and ≈2-fold decreased susceptibility to, respectively, OS and ZA – virus isolates

A/Portugal/17/2009 and A/Portugal/82/2009, carried the NA I223V amino acid substitution (Table 4.7). This substitution is known to confer slightly decreased susceptibility to NAIs in A(H1N1)pdm09 subtype (6-fold OS, 2-fold ZA; see Table 1.2, Literature Review for detail).

The remaining A(H1N1)pdm09 upper outlier viruses contained either a single or two NA amino acid substitutions located within the globular head but at some distance (K432E) or far away (I359M, T381N, G454S) from the active site, or no specific NA amino acid substitution (majority) (Table 4.7; Figure 4.10A). Most A(H1N1)pdm09 lower outlier viruses evaluated through NA sequencing contained also no specific amino acid substitution. Only two OS LM outlier viruses from the pandemic period carried a single (virus isolate A/Portugal/27/2009) or three (virus isolate A/Portugal/01/2010) NA amino acid substitutions, located in the stalk (V53A, T48K) or within the globular head but far away from the active site (V267I, W457G) (Table 4.7; Figure 4.10A). Residue 267 (V267I substitution) belongs to a known antibody epitope of 2009 pandemic N1 NA (CD6 epitope) [26].

The NA Y155H amino acid substitution known to confer (H)RI by OS and ZA in former circulating A(H1N1) viruses was identified in A(H1N1)pdm09 viruses classified as either lower outlier (n=3; Table 4.7) or non-outlier (n=16). This evidenced that its impact on virus susceptibility does not extend to 2009 pandemic A(H1N1) subtype.

The non-outlier virus isolate A/Portugal/28/2009 that, similarly to NA H275Y variant virus A/Portugal/03/2011, was recovered from a patient suspected of having developed drug-resistant influenza during OS therapy (suspected case 1; see Table 3.2, Material and Methods), contained no specific amino acid substitution in NA. The clinical specimen yielding this virus isolate presented an identical NA genetic background.
NA sequencing of the virus population present in clinical specimens collected from the same patient before, during and after antiviral therapy, showed the emergence of NA Y66C amino acid substitution as viral quasispecies after therapy (Y66C/Y mixed virus population). Residue 66 is located in the stalk domain of the protein (Table 4.7).

Table 4.7 Neuraminidase and hemagglutinin amino acid substitutions specific of the A(H1N1)pdm09 viruses classified as phenotypic outlier and/or recovered from a patient under antiviral therapy, and further substitutions associated with neuraminidase inhibitor resistance or decreased susceptibility.

	Influenza season / 2009 pandemic period	Virus isolate	Phenotypic and other relevant information	A(H1N1)pdm09											
				NEURAMINIDASE					HEMAGGLUTININ						
									HA1[a]						HA2 (F Fusion sub-domain)
				Stalk domain	Catalytic domain (globular head)				F´ Fusion sub-domain		R sub-domain				
PHENOTYPIC UPPER OUTLIER	Pandemic period	A/Portugal/17/2009	OS UEo; ZA UMo	-	***I223V***[f]	-	-	-			None				
		A/Portugal/18/2009	OS UMo	-	K432E/K[g]	G454S	-	-	-	V199I	-	-	-		-
		A/Portugal/64/2009	OS UMo			None			N276S	-	-				-
		A/Portugal/66/2009	ZA UMo			None					None				
		A/Portugal/78/2009	OS UMo			None					None				
		A/Portugal/82/2009	OS UEo; ZA UMo	-	***I223V***[f]	-	-	-			None				
		A/Portugal/101/2009	ZA UMo			None					None				
		A/Portugal/114/2009	OS UEo			None			I286T	T288S	-				-
	2010/2011	A/Portugal/03/2011[b,c]	OS UEo, HRI First-passage virus isolate	-	V241I[h]	H275Y	P337S/P[g]	N369K[h]	-	N156D	-	-	-		-
		A/Portugal/07/2011	OS UMo; ZA UEo	-	V241I[h]	N369K[h]	-	-	-	N129D	K153E/K[g]	N156K	G237R		-
		A/Portugal/10/2011	ZA UMo	-	V241I[h]	N369K[h]	T381N	-			None				
		A/Portugal/22/2011	OS UMo	-	V241I[h]	N369K[h]	-	-			None				
	2012/2013	A/Portugal/54/2013	OS/ZA UEo	-	V241I[h]	I359M	N369K[h]	-	-	K163E	-	-	-		-
		A/Portugal/55/2013[d]	OS/ZA UEo Third-passage virus isolate	-	V241I[h]	N369K[h]	-	-	-		-				Y162F
		Matching clinical specimen	Collected before OS therapy	-	V241I[h]	N369K[h]	-	-	-		-				Y162F
PHENOTYPIC LOWER OUTLIER	Pandemic period	A/Portugal/08/2009	OS/ZA LMo	-	Y155H[i]	-	-	-			None				
		A/Portugal/27/2009	OS LMo	V53A	Y155H[i]	-	-	-			None				
		A/Portugal/01/2010	OS LMo	T48K	V267I	W457G/W[g]	-	-	-		-				D174N
		A/Portugal/02/2010	OS LMo	-	Y155H[i]	-	-	-			None				
	2010/2011	A/Portugal/27/2011	ZA LMo	-	V241I[h]	N369K[h]	-	-	H51Y/H[g]	-	-				D109G
ASSOCIATED WITH ANTIVIRAL USE	Pandemic period	A/Portugal/28/2009[b]	Third-passage virus isolate	-	Y155H[i]	-	-	-			None				
		Matching clinical specimen	Collected during OS therapy (starting date not available)	-	Y155H[i]	-	-	-			None				
	2012/2013	Clinical specimen[e]	Collected on day 7 of OS therapy	-	V241I[h]	N369K[h]	-	-	-		-				Y162F
		Clinical specimen[e]	Collected one day after the end of OS therapy	Y66C/Y[g]	V241I[h]	N369K[h]	-	-	-		-				Y162F

HRI: Highly reduced inhibition; LMo: Lower mild outlier; OS: Oseltamivir; R: Receptor binding; UEo: Upper extreme outlier; UMo: Upper mild outlier; ZA: Zanamivir

Colour legend:

Associated with HRI by OS and peramivir (PER) *in vitro* and know to cause clinical resistance to OS - influenza N1 neuraminidase (NA) viruses

Associated with a normal inhibition ≥2-fold by NA inhibitors (NAIs) *in vitro*, confirmed by reverse genetics – influenza A(H1N1)pdm09 viruses

Potential compensatory substitution of the fitness deficits caused by NA H275Y substitution in A(H1N1)pdm09 viruses

[a] Position numbering without the N-terminal signal sequence (amino acid residues 1-17); [b] Recovered from a patient suspected of having developed a drug-resistant influenza virus infection during OS therapy; [c] NA H275Y amino acid substitution detected in the entire virus population of the clinical specimen (H275Y screening result provided by the Hospital Curry Cabral); clinical specimen collected during therapy but not possible to specify in which day (starting date not available); [d] From a case of A(H1N1)pdm09 virus infection associated with antiviral therapy but isolated from the clinical specimen collected before OS use; [e] Post-treatment clinical specimens from the same case of A(H1N1)pdm09 virus infection as virus isolate A/Portugal/55/2013; [f] Is also known to enhance the level of reduced susceptibility conferred by H275Y, alone (PER) or in combination with N295S (OS, ZA) - synergistic amino acid substitution (see Table 1.2, Literature Review); [g] Mixed virus population; [h] Identified in all A(H1N1)pdm09 viruses tested and in most reference A(H1N1)pdm09 viruses, from 2010/2011 onwards; [i] Also identified in approximately half (15/32; including A/Portugal/28/2009) of the A(H1N1)pdm09 non-outlier viruses from the pandemic period and in the only A(H1N1)pdm09 non-outlier virus from 2012/2013; exceptionally indicated as it is known to confer (H)RI by OS and ZA in former circulating A(H1N1) viruses.

The amino acid substitutions were identified and are displayed as described in Table 4.4 (2009 pandemic N1 and H1 numbering). The analysis involved a total of 253 NA and 243 HA sequences from tested (n=214 NA; n=205 HA; (+4 clinical specimens)) and reference (n=35 NA; n=34 HA) A(H1N1)pdm09 viruses (detailed information in Tables S4.1 and S4.2, Supplementary data). The location of the NA structural domains in the 2009 pandemic N1 monomer was based on Colman [8] and da Silva *et al.* [27]; while the location of the HA structural sub-domains in the 2009 pandemic H1 monomer was established according to Gamblin *et al.* [11] and Yang *et al.* [28]. The amino acid substitutions occurring at residues belonging to the NA active site (based on Colman *et al.* [14]) are underlined. Detailed information on the NA amino acid substitutions associated with NAI resistance or decreased susceptibility can be found at Table 1.2, Literature Review.

The upper outlier virus isolate A/Portugal/07/2011 with decreased susceptibility to both OS and ZA (2010/2011), carried the HA N129D, K153E, N156K and G237R amino acid substitutions in the receptor binding HA1 sub-domain (Table 4.7). All substitutions except G237R are located closely to the HA RBS (Figure 4.10B). According to Skowronski *et al.* [20], residues 153 and 156 belong to the antigenic site Sa, while residue 237 is part of the antigenic site Ca1. The presence of HA K153E amino acid substitution as viral quasispecies (K153E/K mixed virus population) is in agreement with its known association to virus adaptation to cell culture [29]. HA K153E substitution has also been selected *in vitro* after serial passage under laninamivir (LAN) drug pressure (see section 1.5.2.1.2, Literature Review for detail), but its effect on NAI susceptibility is still unknown.

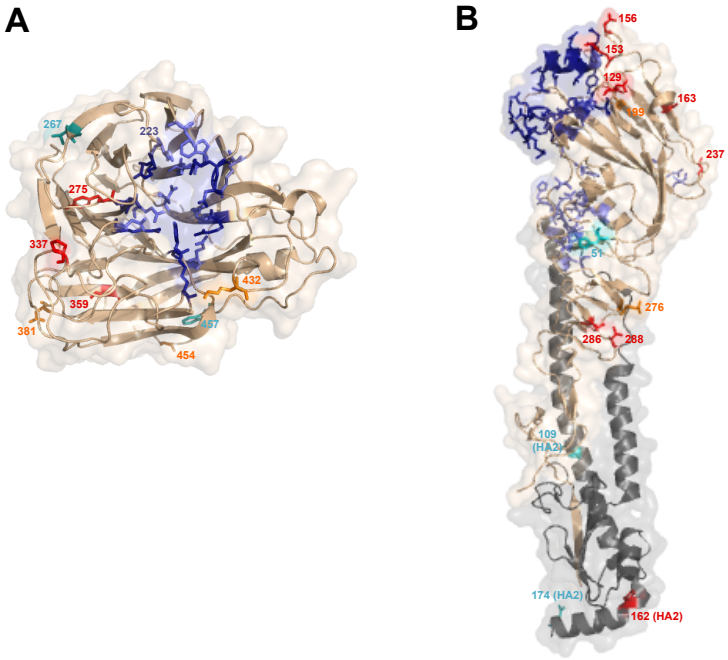


Figure 4.10 Mapping of the neuraminidase **(A)** and hemagglutinin **(B)** residues on which were detected the amino acid substitutions specific of the A(H1N1)pdm09 phenotypic outlier viruses, onto the three-dimensional structure of the protein.

The figure was generated and annotated as described in Figure 4.7, using the structure of the neuraminidase (NA) globular head (PDB ID: 3NSS, only one globular head of the two described in the file is shown) (**panel A**) and of the hemagglutinin (HA) (PDB ID: 3LZG, only one monomer from one of the two HA trimers described in the file is shown) (**panel B**) from the reference A(H1N1)pdm09 virus A/California/04/2009. The residues forming the RBS in the 2009 pandemic H1 HA are based on Yang *et al.* [30]. NA active site residues are not coloured according to the phenotypic outlier category of the virus carrying the substitution, maintaining the colour assigned to the corresponding type of active site residue (functional or framework). **Panel A**: Residue 275 associated with clinical resistance to oseltamivir is also indicated.

NA H275Y variant virus A/Portugal/03/2011 contained the HA N156D amino acid substitution located closely to the RBS. All other HA amino acid substitutions identified in either the remaining A(H1N1)pdm09 upper outlier viruses or in the lower outlier viruses evaluated through HA sequencing, were located at some distance (V199I) or far away from the RBS (Table 4.7; Figure 10.B). The HA H51Y amino acid substitution identified in an LM outlier virus from 2010/2011 - virus isolate A/Portugal/27/2011 (H51Y/H), occurred, however, near the putative second sialic acid binding site on HA (Figure 10.B). Residue 163 in which was detected the single HA amino acid substitution of the UE outlier virus isolate A/Portugal/54/2013 (K163E) belongs to the antigenic site Sa [20].

No specific HA amino acid substitution was identified in the non-outlier virus isolate A/Portugal/28/2009 with known association to antiviral drug use and in its matching clinical specimen (Table 4.7). Also, no HA amino acid substitution emerged during or after OS therapy of the patient from which were collected the clinical specimens from before, during and after antiviral use (HA Y167F amino acid substitution identified in all three specimens).

HA D222G amino acid substitution known to confer decreased NAI susceptibility in former circulating A(H1N1) viruses (D221G in seasonal H1 numbering; see section 1.5.2.1.2, Literature Review) was identified in an A(H1N1)pdm09 non-outlier virus from 2012/2013 – virus isolate A/Portugal/53/2013. In current circulating 2009 pandemic A(H1N1) viruses, this HA substitution has only be selected *in vitro* after serial passage under LAN drug pressure (detailed in section 1.5.2.1.2, Literature Review), with its effect on NAI susceptibility remaining unknown.

4.1.4.2.2 Evolutionary relationships of neuraminidase and hemagglutinin genes

4.1.4.2.2.1 Former seasonal influenza N1 and H1 genes

Phylogenetic comparison of seasonal influenza N1 genes showed that the OS UE outlier viruses showing HRI from 2007/2008 were closely related to those from 2008/2009, clustering into a group within genetic clade 2B defined by the NA H275Y amino acid substitution (Figure 4.11A). The additional presence of the NA D354G amino acid

substitution in all these NA H275Y variant viruses (not evidenced in the tree; see section 4.1.4.2.1.1 above), indicated that all belonged to the Northern European OS-resistant lineage in clade 2B [31]. The HRI NA H275Y variant viruses from 2008/2009 clustered into a further subgroup defined at only nucleotide level, by the NA T234G/A nucleotide substitution (Figure 4.11A). Most of these viruses were very closely related to each other, with some of them presenting 100% identical sequences (e.g. A/Lisboa/21/2009 and A/Lisboa/23/2009). Virus isolates A/Lisboa/07/2009 and A/Lisboa/29/2008 and A/Lisboa/03/2009, these two latter grouped by the NA T435R amino acid substitution, were more closely related to, respectively, the reference viruses A/Hong Kong/3192/2008 and A/England/557/2007.
All other seasonal A(H1N1) phenotypic outlier viruses (UM, LM and LE) were closely related to the non-outlier viruses from the same influenza season (Figure 4.11A).

A more extensive phylogenetic analysis of influenza virus NA genes was further performed in seasonal A(H1N1) subtype, including all NA sequences of worldwide circulating NA H275Y variant viruses available at GISAID EpiFlu™ and NCBI Influenza Virus Resource databases (n=585 by January 2012; sequences were comprised in the seasonal A(H1N1) dataset created during this PhD work to study the selective pressure acting on human influenza NA; Chapter 6). This more extensive analysis made clear the subtle separation previously observed for the NA H275Y viruses from 2008/2009 that now clustered within three subgroups: (1) most viruses clustered with NA H275Y viruses from Europe and North America (closest) and from Africa; (2) virus isolate A/Lisboa/07/2009 clustered with NA H275Y viruses from particularly Eastern Asia (closest) but also from Europe and the Americas; and (3) virus isolates A/Lisboa/29/2008 and A/Lisboa/03/2009 grouped with NA H275Y viruses from the Americas (Figure 4.11B). It also revealed a similar separation into three subgroups for the NA H275Y viruses from 2007/2008, with the virus isolates A/Lisboa/27/2008 and A/Lisboa/28/2008 clustering together. A/Lisboa/02/2008, A/Lisboa/11/2008 and A/Lisboa/20/2008 clustered with NA H275Y viruses from all worldwide continents except Oceania, while the virus isolate A/Lisboa/03/2008 grouped with NA H275Y viruses from Europe and South America (Figure 4.11B).

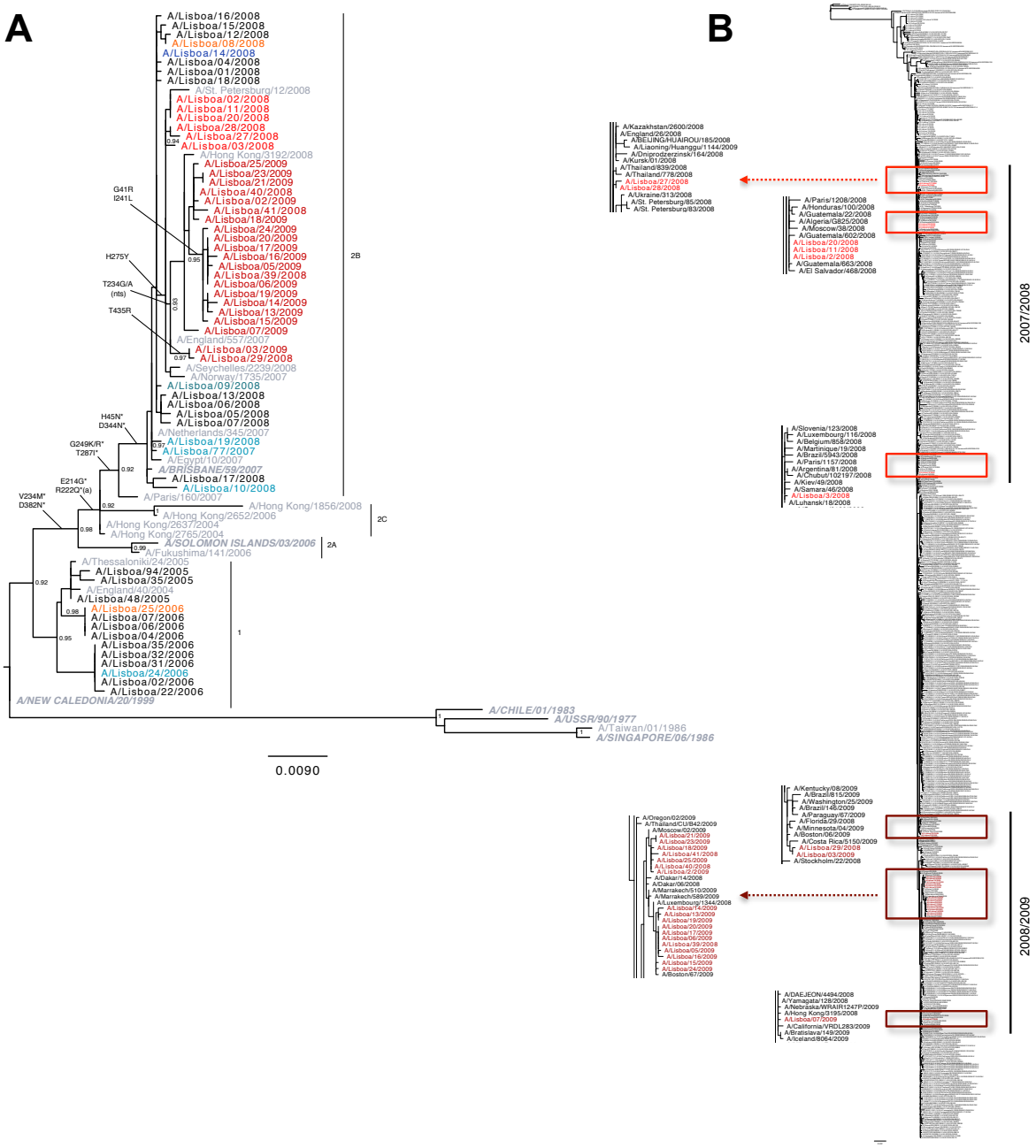


Figure 4.11 Evolutionary relationships among the neuraminidase genes of former seasonal A(H1N1) tested and reference viruses **(A)** and additionally of all worldwide NA H275Y former seasonal A(H1N1) variant viruses with a sequence publicly available **(B)**.

nts: nucleotides; (a) Also present in the reference virus A/USSR/90/1977 (outgroup)

Phylogenetic trees were constructed by maximum-likelihood method in the PhyML3.0 platform available in SeaView, using the nucleotide substitution model GTR+I+Γ4 (General Time Reversible (GRT) model assuming a proportion of invariable sites (I) and with a 4-category gamma distribution (Γ4) for rate variation among sites), previously determined in jModelTest. SPR and NNI rearrangement operations were used to optimise tree topology, while SH-like aLRT method was used to estimate branch support. Tested viruses are colour-coded according to their phenotypic outlier category as follows: red - upper extreme (UE) outlier, using two different shades of red to distinguish the viruses from 2007/2008 (light red) and 2008/2009 (dark red); orange - upper mild (UM) outlier; turquoise blue - lower mild (LM) outlier; blue - lower extreme (LE) outlier. If the virus belonged to different outlier categories in oseltamivir and zanamivir susceptibility testing, it was considered the category more above in the following order: UE>UM>LE>LM. Worldwide reference viruses are highlighted in grey, including the recommended vaccine viruses that are further indicated in capital letters and in bold and italic. Earlier reference viruses were used to root the

(Footnotes Figure 4.11 cont.)
phylogenetic trees (outgroup) and provide directionality to the evolutionary histories. Detailed information on the reference nucleotide sequences used can be found at Table S4.2, Supplementary data. The accession number of the nucleotide sequences of the viruses tested shared through public-access databases is also available in Supplementary data (Table S4.1). **Panel A**: SH-like aLRT branch support values equal or higher than 0.90 are indicated in the tree. The genetic clades to which the different viruses belong are also indicated on the right side. Four genetic clades are defined for former circulating A(H1N1) viruses (based on hemagglutinin gene), designated as 1, 2A, 2B and 2C and represented by, respectively, A/New Caledonia/20/1999, A/Solomon Islands/03/2006, A/Brisbane/59/2007 and A/Hong Kong/2652/2006 reference viruses [32]. Only the amino acid substitutions relevant for the interpretation of phenotypic susceptibility data are indicated in the tree. All substitutions were identified against the seasonal N1 consensus sequence, except those highlighted with an asterisk (*) that were identified against the sequences of the viruses not comprised in that specific evolutionary clade or group. **Panel B**: included 585 additional neuraminidase (NA) gene sequences from NA H275Y former seasonal A(H1N1) variant viruses circulating worldwide. These sequences were available at GISAID EpiFlu™ and NCBI Influenza Virus Resource databases by January 2012 and were comprised in the former seasonal A(H1N1) dataset created during this PhD work to study the selective pressure acting on influenza NA (Chapter 6).

Of note, the change in the natural *in vitro* susceptibility of seasonal A(H1N1) viruses to OS (decrease) and ZA (increase) between 2005/2006 and 2007/2008 matched a change in the genetic clade to which the circulating viruses belonged. As shown in Figure 4.11A, all non-outlier viruses from 2004/2005 and 2005/2006 belonged to the genetic clade 1, represented by the reference virus A/New Caledonia/20/1999 (NC/99), while those from 2007/2008 onwards fell into the genetic clade 2B, represented by A/Brisbane/59/2007 (BR/07). Six NA amino acid substitutions distinguished the BR/07-like from previous NC/99-like viruses: E214G, R222Q, V234M, G249K, T287I and D382N. According to the 1918 N1 NA structure, all these amino acid substitutions occurred at the surface of the globular head, except R222Q and G249K substitutions that occurred closely to the active site (Figure 4.12A). Residue 222 lies adjacently to the framework active site residue 223, while residue 249 belongs to the 243-251 surface loop that is a potential antigenic site of seasonal N1 NA [33]. Moreover, the arginine (R) to glutamine (Q) amino acid substitution at residue 222 is known to significantly increase NA binding affinity to its substrate [34,35] (potential fitness-compensatory substitution). The NA D344N amino acid substitution also known to significantly increase NA binding affinity to its substrate [34-36] and, additionally, to OS and ZA [37], was present in all BR/07-like viruses except A/Paris/160/2006 (Figure 4.11A), occurring closely to the active site (Figure 4.12A).

Regarding the evolutionary relationships of seasonal influenza H1 genes (HA1 subunit), all phenotypic outlier viruses were closely related to the non-outlier viruses from the same influenza season (Figure S4.1A, Supplementary data). The OS UE outlier viruses showing HRI (NA H275Y variant viruses) fell into the genetic clade 2B, with those from

2008/2009 continuing to cluster together like in NA, but with no amino acid or nucleotide substitution defining the subgroup. The virus isolates A/Lisboa/29/2008 and A/Lisboa/03/2009 remained also grouped (HA G152E) and positioned more apart from the other UE outlier viruses comprising the subgroup. Among these was included the virus isolate A/Lisboa/07/2009, with all sharing HA S141N and G185A amino acid substitutions (Figure S4.1A, Supplementary data).

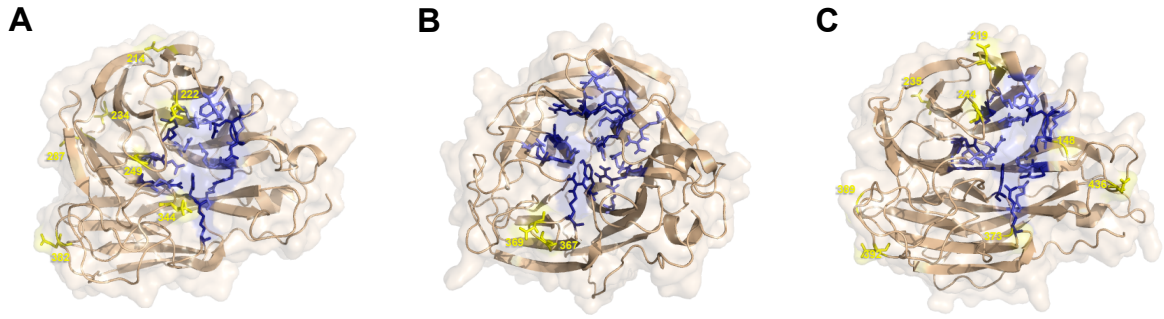


Figure 4.12 Mapping of the neuraminidase residues on which occurred the amino acid substitutions differentiating former seasonal influenza A(H1N1) **(A)** or influenza A(H3N2) **(B)** genetic clades and influenza B co-circulating lineages **(C)**, onto the three-dimensional structure of the protein.

The figures were generated and annotated as described in Figure 4.7, except the residues under study that are coloured in yellow. **Panel A**: amino acid substitutions differentiating clade 2B A/Brisbane/59/2007-like viruses from clade 1 A/New Caledonia/20/1999-like viruses. Residue 344 is also indicated, as NA D344N amino acid substitution was present in all A/Brisbane/59/2007-like viruses except A/Paris/160/2006. The former seasonal N1 neuraminidase (NA) structure used was the same as in Figure 4.7A. **Panel B**: amino acid substitutions differentiating Victoria/208 clade viruses from previously circulating Brisbane/10, Wisconsin/67 and California/07 clade viruses. It was used the same N2 NA structure as in Figure 4.8A. **Panel C**: amino acid substitutions characteristic of B/Victoria-lineage viruses, compared to the B/Yamagata-lineage NA consensus sequence. The influenza B/Victoria-lineage NA structure used was the same as in Figure 4.9A.

4.1.4.2.2.2 Influenza A(H3N2), B and A(H1N1)pdm09 virus neuraminidase and hemagglutinin genes

Phylogenetic comparison of influenza A(H3N2), B and A(H1N1)pdm09 virus NA (Figure 4.13) and HA (Figure S4.1B-D, Supplementary data) genes showed that the phenotypic outlier viruses were closely related to the non-outlier viruses from the same influenza season or period and, for type B influenza, also lineage. The A(H3N2) UM outlier virus A/Portugal/29/2012 clustered within a different group (group 3B) than the non-outlier viruses from 2011/2012 (group 6), but probably due to the high diversity of genetic groups within Victoria/208 clade.

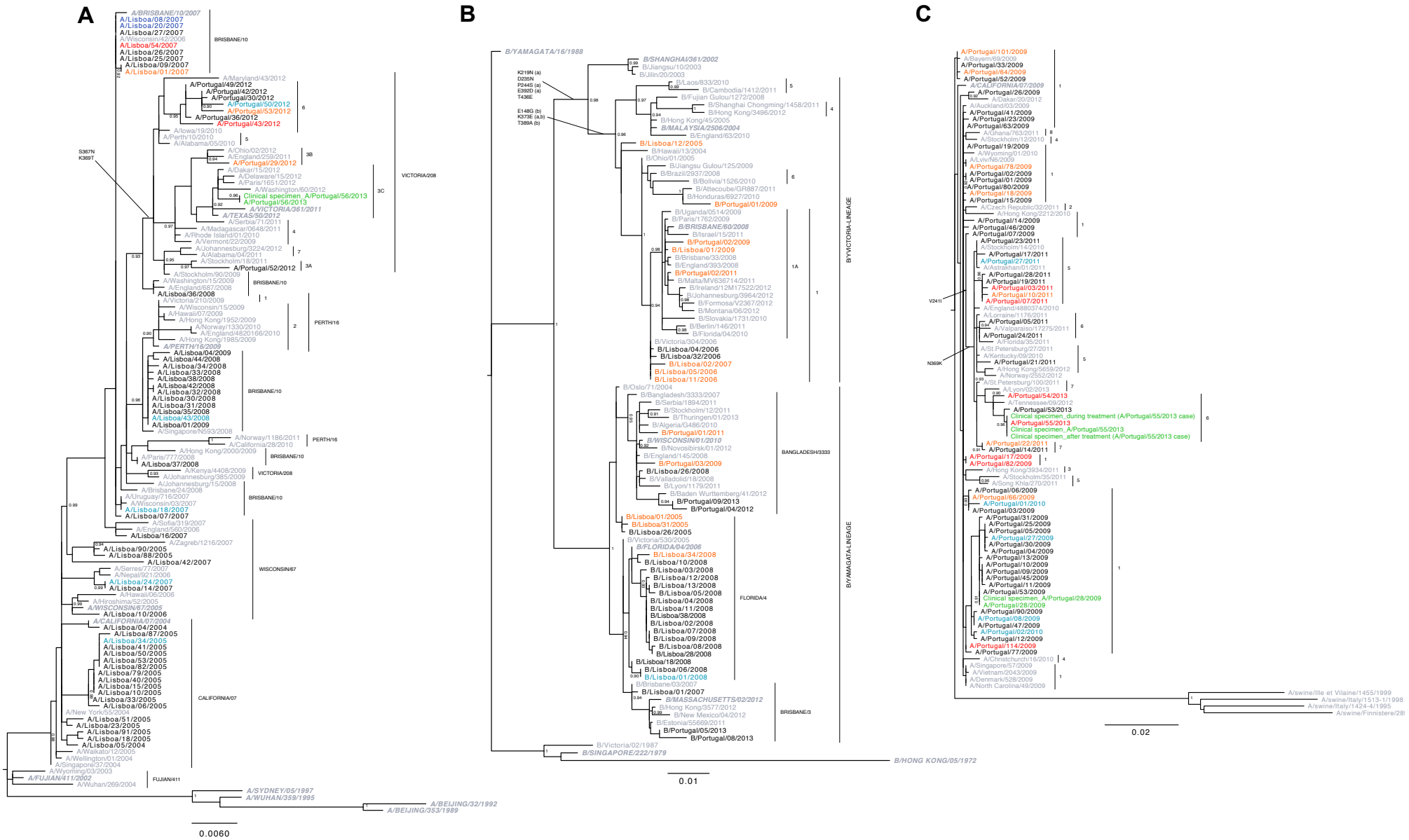


Figure 4.13 Phylogenetic comparison of influenza A(H3N2) **(A)**, B **(B)** and A(H1N1)pdm09 virus **(C)** neuraminidase genes.

(Footnotes Figure 4.13)

Panel B: (a) also present in all or nearly all outgroup viruses; (b) only not present in one or two (T389A) reference B/Victoria-lineage viruses.

Neuraminidase (NA) phylogenetic trees were constructed and annotated as described in Figure 4.11 (panel A), with the upper extreme outlier viruses being just coloured in light red. The relevant N2 (**panel A**) and 2009 pandemic N1 (**panel C**) amino acid substitutions indicated in the tree were identified against the corresponding consensus sequence, while the amino acid substitutions characteristic of B/Victoria (VIC) - lineage viruses indicated in the influenza B NA tree (**panel B**) were identified against the consensus sequence of B/Yamagata (YAM) - lineage viruses. **Panel A**: the six genetic clades considered for circulating A(H3N2) viruses include three earlier clades - Fujian/411, California/07 and Wisconsin/67, defined by the vaccine viruses A/Fujian/411/2002, A/California/07/2004 and A/Wisconsin/67/2005; and the three recent clades defined by WHO (based on hemagglutinin (HA) gene) - Brisbane/10, Perth/16 and Victoria/208, represented by the reference viruses A/Brisbane/10/2007, A/Perth/16/2009 and A/Victoria/208/2009 (the first two also vaccine viruses) [38]. Perth/16 and Victoria/208 genetic clades are further divided according to the established genetic groups: 1 and 2 (Perth/16); 3A, 3B, 3C, 4, 5, 6 and 7 (Victoria/208) [39]. **Panel B**: the HA genetic clades defined by WHO [40] for each influenza B virus lineage are indicated in the tree, including: clades 1 (or Brisbane/60, represented by the vaccine virus B/Brisbane/60/2008), 4, 5 and 6 for B/VIC lineage, in which clade 1 is further divided into the established genetic groups 1A and 1B [41]; and the Florida/4, Brisbane/3 (or clade 2) and Bangladesh/3333 (or clade 3) clades for B/YAM lineage, represented by the reference viruses B/Florida/04/2006 (vaccine virus), B/Brisbane/3/2007 and B/Bangladesh/3333/2007. **Panel C**: the HA genetic groups defined by WHO [41] for circulating A(H1N1)pdm09 viruses are indicated in the tree, including 8 genetic groups designated from 1 to 8 and represented by the reference viruses A/California/07/2009 (vaccine virus; 1), A/Czech Republic/32/2011 (2), A/Hong Kong/3934/2011 (3), A/Christchurch/16/2010 (4), A/Astrakhan/01/2011 (5), A/St Petersburg/27/2011 (6), A/St Petersburg/100/2011 (7), and A/Norway/2552/2012 (8).

Influenza A(H3N2) virus NA phylogenetic tree also showed that a change to a new drift variant may have been at the origin of the decrease observed in the natural *in vitro* susceptibility of circulating viruses to OS and ZA, between 2008/2009 and 2011/2012. All A(H3N2) non-outlier viruses from 2011/2012 onwards belonged to the Victoria/208 genetic clade, while those circulating previously belonged to either Brisbane/10, Wisconsin/67 or California/07 genetic clades (Figure 4.13A). Two NA amino acid substitutions located closely to the active site - S367N and K369T, distinguished the A/Victoria/208/2009-like viruses from the viruses belonging to these three previous clades (Figure 4.13A; Figure 4.12B).

The influenza B UM outlier virus B/Lisboa/12/2005 revealed to be an inter-lineage reassortant, possessing a NA gene from the B/VIC-lineage (Figure 4.13B) and having a B/YAM-like HA gene (Figure S4.1C, Supplementary data). As influenza B lineage differentiation was based on the antigenic and/or genetic characteristics of HA, this virus isolate was mistakenly analysed as a B/YAM-lineage virus in NA inhibition assay. No genetic support was found for the decrease observed in the natural *in vitro* OS susceptibility of circulating influenza B viruses after 2004/2005 and then further in the pandemic period. However, 8 NA amino acid substitutions distinguished the B/VIC-

lineage viruses that overall exhibited a lower natural susceptibility to ZA and, although less evidently, to OS, compared to B/YAM-lineage viruses. Specifically: NA E148G, K219N, D235N, P244S, K373E, T389A, E392D and T436E substitutions (Figure 4.13B). Four of these substitutions - E148G, K219N, P244S, K373E, are closely to the active site (Figure 4.12C).

The two NA I223V A(H1N1)pdm09 OS UE/ZA UM outlier viruses A/Portugal/17/2009 and A/Portugal/82/2009 presented 100% identical NA (Figure 4.13C) and HA (Figure S4.1D, Supplementary data) gene sequences. Based on patient epidemiological and clinical data, these two outlier viruses were also geographically and temporally closely related. No genetic support was found for the slight decrease in the natural *in vitro* susceptibility of circulating A(H1N1)pdm09 viruses to OS between the pandemic period and 2010/2011. A(H1N1)pdm09 non-outlier viruses from 2010/2011 belonged to new genetic groups (groups 5, 6 and 7), but only NA N369K amino acid substitution differentiated these non-outlier viruses from those circulating during the pandemic period (genetic group 1) (Figure 4.13C). NA N369K variant viruses are known to exhibit increased (and not decreased) affinity for the substrate (potential fitness-compensatory substitution) [42].

4.1.5 Influenza Antiviral Prescription on Influenza-like Illness Patients (complementary study)

Between 2005/2006 and 2008/2009, influenza antivirals were prescribed at very low frequencies to the influenza-like illness (ILI) patients presenting to healthcare systems covered by the National Influenza Surveillance Programme. The frequencies varied from 1.0% (6/600) in 2008/2009 to 3.1% (7/226) in 2007/2008 (Figure 4.14), not differing significantly over time (Pearson Chi-Square Test, $p = 0.191$). Antiviral information was missing for more than 650 of the total 2290 ILI cases notified during the period studied, which were excluded from the analysis.

Virtually all cases of ILI reporting antiviral prescription were received through the network of emergency units (27/30; 90.0%). Influenza antivirals were mainly prescribed to patients within the 15–44 years age group (22/30; 73.3%), with no

underlying risk condition. Four (13.3%) of the patients to whom antivirals were prescribed had previously been vaccinated for influenza but no information was available regarding vaccination date. OS was the antiviral most prescribed, having been identified in 19 (90.5%) of the 21 notifications on which the antiviral was specified. However, approximately half (14; 46.7%) of the ILI patients to whom antivirals were prescribed tested negative for influenza virus infection.

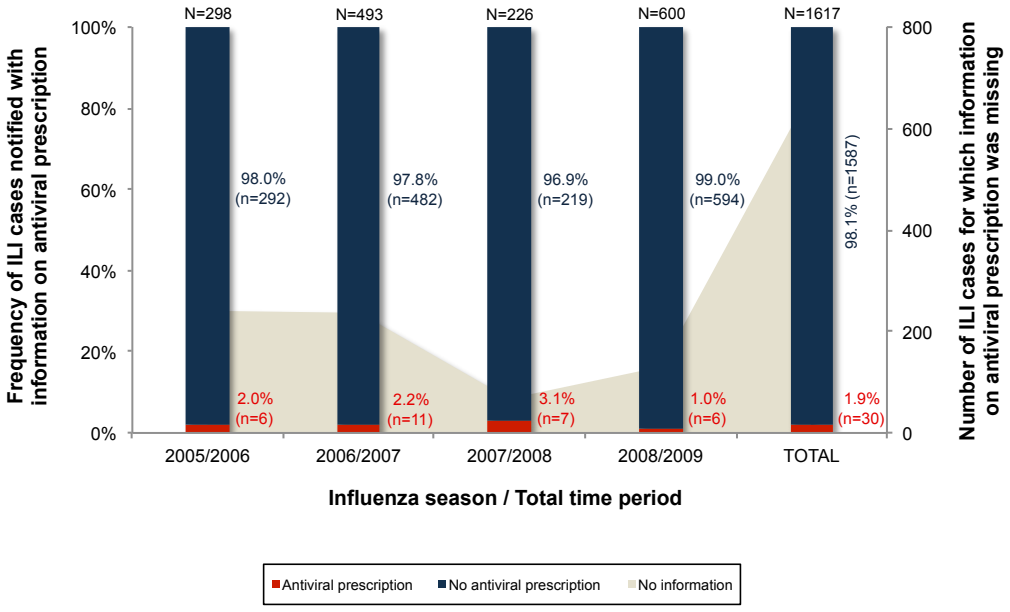


Figure 4.14 Distribution of the cases of influenza-like illness notified between 2005/2006 and 2008/2009 in the context of the National Influenza Surveillance Programme, according to antiviral prescription.

ILI: Influenza-like illness

The number of ILI cases notified with information on antiviral prescription is displayed at the top of each stacked column. At the left side are specified the frequencies of antiviral prescription and no antiviral prescription, using the same colour as the section in the column. The corresponding number of cases is shown below under brackets.

4.2 DISCUSSION

This PhD work established the capacity and capability to comprehensively evaluate and monitor influenza antiviral susceptibility at national level. Human influenza viruses circulating in Portugal were for the first time characterized regarding their susceptibility to the three antivirals approved nationally for clinical use - AMA (M2 inhibitor), and OS and ZA (NAIs). Antiviral susceptibility testing of circulating viruses started in 2008 but

went back 3 influenza seasons (until 2004/2005; retrospective study) to match the beginning of influenza antiviral susceptibility surveillance in Europe (2004; VIRGIL network). At this time, only a very limited number of European countries were fully testing and reporting influenza antiviral susceptibility, contributing to the overall data generated by the VIRGIL network [43]. Also, only a small proportion of the viruses circulating globally was being tested for antiviral susceptibility [7]. The first national antiviral susceptibility data generated through this study was therefore extremely valuable for the surveillance carried out at both European and global level. Antiviral susceptibility testing activities continued until 2012/2013, covering a total of 8 influenza seasons plus the 2009 pandemic period. Influenza viruses circulating in both community and hospitalized settings were tested, with these later including viruses recovered from patients undergoing antiviral therapy either or not suspected of having developed drug-resistant influenza.

4.2.1 Influenza Antiviral Susceptibility Testing Platform

The technological platform established for influenza antiviral susceptibility testing comprises a genotypic platform for evaluation of M2 inhibitor susceptibility (complete cross-resistance between AMA and RIM[6]); and a twofold phenotypic-genotypic platform combined, whenever possible, with patient clinical data, for evaluating the susceptibility to OS and/or ZA. Notably, the two phenotypic NA inhibition assays implemented in the platform are suitable to any novel NAI targeting the NA active site, as it is the case of the two recently available drugs PER and LAN, for now only approved in very limited markets [44-46], and of the investigational tamiphosphor (pre-clinical stage) [47]. Genotypic testing through full-length sequencing of NA and HA genes can also extend to any novel NAI, independently of its target. The different complexity of the platforms implemented for M2 inhibitor drug class and OS and ZA reflects the different knowledge on the mechanisms underlying the development of clinical resistance. The genetic basis for clinical resistance to M2 inhibitors is well characterized (7 molecular markers of resistance established), while NA H275Y is the only amino acid substitution known to confer clinical resistance to NAIs, specifically to OS and solely in N1 NA influenza viruses (seasonal A(H1N1), A(H1N1)pdm09 and A(H5N1)) [48]. Several amino acid substitutions in NA, as well as two amino acid substitutions at position 221 of the HA1 subunit of

seasonal H1 HA (D221G/N), have been associated with reduced NAI susceptibility *in vitro*, but its clinical impact is less clear or completely unknown [5,49]. Also, the relationship between reduced NAI susceptibility *in vitro* (phenotype) and genotypic background (genotype) is not always predictable, remaining one of the major challenges in influenza antiviral susceptibility testing [50]. An influenza virus exhibiting reduced susceptibility *in vitro* may retain the genetic characteristics of a fully susceptible virus or carry a still unknown amino acid substitution conferring RI or HRI by the drug.

Phenotypic methodologies accounting for HA-mediated changes in susceptibility were not implemented, as those available were either not recommended (cell-culture based assays) or even not yet standardized (red blood cell elution assays) [51,52]. In the absence of phenotypic data, the genetic analysis of virus HA to evaluate NAI susceptibility is limited to the screening of the two HA amino acid substitutions known to confer reduced susceptibility and, when applicable, the comparison of the HA of the virus population present before and after treatment.

4.2.2 Influenza A Virus Resistance to M2 Protein Inhibitors

Influenza A(H3N2) viruses resistant to M2 inhibitors began to circulate in Portugal in 2005/2006. M2 inhibitor resistance was initially estimated in 100% (single virus isolated), changing to 74.5% in 2006/2007 and then settling at 100% during the following influenza seasons analysed (2008/2009, 2011/2012 and 2012/2013). All resistant viruses carried the S31N amino acid substitution in the M2 protein, with three viruses from 2008/2009 further carrying the V27A substitution (double V27A/S31N mutant viruses). The beginning of M2 inhibitor resistance in Portugal matched the emergence and global spread of a new lineage of drug-resistant S31N variant viruses, designated as N-lineage [53]. In the origin of this new lineage is a 4+4 genome reassortment event in early 2005, involving the drug-resistant viruses circulating in South-East Asia in the presence of drug-selective pressure, specifically those circulating in Hong Kong as recently evidenced by Nelson *et al.* [54]. The presence of beneficial amino acid substitutions in the novel HA acquired by reassortment (A/Wisconsin/67/2005-like HA), with a hitchhiking effect on M2 S31N amino acid substitution, has been considered as the most probable explanation for the global spread of M2 inhibitor-resistant N-lineage viruses [53,54]. Drug-selective pressure had apparently no role in this global spread, with S31N

resistant viruses spreading around the world independently of the presence or absence of selective pressure from drug use. Portugal belongs to this latter group. The use of AMA (only M2 inhibitor licensed; Parkadina®) has been essentially for the treatment of Parkinson´s disease and not influenza A virus infections, with the drug belonging to the pharmacotherapeutic group of anti-Parkinson drugs, dopamine agonists (www.infarmed.pt).

The frequency at which A(H3N2) M2 inhibitor-resistant viruses were detected in Portugal was in accordance with the frequencies reported by other national and global influenza antiviral susceptibility surveillance studies. These ranged from 42.7% to 100% in 2005/2006 [55-57] and from 78% to 89.6% between 2007 and 2007/2008 [58,59], settling at 100% from 2008 onwards [59,60]. The M2 V27A/S31N combination found in three resistant viruses has been identified worldwide, representing the second most frequent resistant genotype (after S31N) of influenza H3 HA viruses circulating between 1968 and 2013 [61]. Moreover, the frequency at which was detected at national level (5.7%) was very similar to that at which was detected globally among these H3 HA viruses (5.4%) [61]. The mechanisms underlying the emergence of this double M2 S31N/V27A resistant variant remain, however, unknown.

The natural resistance exhibited by recently emerging A(H1N1)pdm09 viruses to M2 inhibitors (M2 S31N) was inherited from the parental Eurasian avian-like swine A(H1N1) virus that since 1989 is characterized for being resistant to this antiviral drug class [62,63].

No M2 inhibitor resistance was observed in seasonal influenza A(H1N1) subtype. Further analysis of the evolutionary relationships among seasonal H1 HA genes, carried out in the context of NAI genotypic susceptibility testing, confirmed that none of the viruses evaluated through HA sequencing belonged to the M2 drug-resistant HA clades 2A and 2C [59]. The viruses from 2004/2005 and 2005/2006 clustered into clade 1, while those from 2007/2008 and 2008/2009 belonged to the clade 2B.

M2 inhibitor susceptibility testing is no longer considered a priority [64]. Naturally resistant 2009 A(H1N1) pandemic viruses replaced seasonal A(H1N1) viruses during 2009/2010 and 2010/2011, resulting in an overall M2 inhibitor resistance among circulating influenza A viruses [65]. The rapid and unpredictable nature of influenza virus evolution makes however important to continue monitoring virus susceptibility to M2

inhibitors, even more in a context of no drug-selective pressure for more than 5 years (M2 inhibitor use not recommend by WHO since 2010 [66]). Given the very limited repertoire of influenza antivirals effective against currently circulating viruses, it is very important to timely detect any change in the susceptibility to M2 inhibitors.

4.2.3 Influenza Virus Susceptibility to Oseltamivir and Zanamivir

Virtually all human influenza viruses circulating in Portugal from 2004/2005 to 2012/2013, except those belonging to seasonal A(H1N1) subtype, were sensitive to both NAIs OS and ZA. Overall frequencies ranged from 91.2% to 98.6% across the different influenza virus types and subtypes and NAI drug, including both phenotypic non-outlier and lower outlier viruses. The frequency of seasonal A(H1N1) viruses sensitive to OS was lower but still high (68.8%), while an overall susceptibility was observed to ZA. Similar results were obtained in other antiviral susceptibility surveillance studies carried out at national [67-70] and worldwide level [71-73] during the time period covered by this study. According to these, 88.3% to 99.9% of the circulating human influenza viruses were sensitive to OS and/or ZA, with the lower limit decreasing to 76.4% when including the OS-sensitive seasonal A(H1N1) viruses. Two of the studies were conducted in Japan [69,70], which alongside with USA is distinguished by a high use of NAIs, specifically OS. All other worldwide countries have been characterized by a relatively low use of NAIs, with an exceptional higher use during the 2009 influenza pandemic [50]. This was well evidenced in the antiviral prescription data obtained in this study, according to which influenza antivirals (90% OS) were only prescribed to ≈2% of the ILI patients presenting to healthcare systems covered by the National Influenza Surveillance Programme in Portugal, from 2005/2006 to 2008/2009. This extremely low prescription rate reflected the national public health policy for antiviral therapy in seasonal influenza that, at that time, was even more restrictive than now. Antiviral use was only recommended for patients with underlying risk conditions or in very particular situations that included clusters of influenza virus infection, cases of infection with highly pathogenic avian influenza A(H5N1) viruses and circulation of vaccine-mismatched influenza viruses [74,75]. Now, it is further recommended to all cases of severe or progressive disease, hospitalized patients and health care professionals infected with influenza [76].

4.2.3.1 NA H275Y Oseltamivir-Resistant Former Seasonal and 2009 Pandemic A(H1N1) Viruses

Resistance to OS was detected in both former and current circulating A(H1N1) viruses (HRI phenotype, NA H275Y substitution), but at two completely different scales. Approximately 21% of the seasonal A(H1N1) viruses circulating during 2007/2008 showed to be resistant, with the frequency increasing to 100% in the following influenza season (2008/2009). Only a single A(H1N1)pdm09 virus from 2010/2011, recovered from an immunocompromised pregnant women hospitalized with severe influenza illness and suspected of having developed drug-resistant influenza during OS therapy, exhibited resistance to the drug - virus isolate A/Portugal/03/2011.

Similar frequencies of OS-resistant seasonal A(H1N1) viruses were obtained in several other worldwide countries, resulting from the emergence and worldwide spread of a fit and transmissible NA H275Y OS-resistant A(H1N1) variant during 2007/2008 with no relation to the country OS usage [72,77,78]. This OS-resistant variant continued to spread globally from 2008 onwards, rapidly outcompeting the drug-sensitive counterparts from circulation, so that, by the end of 2008/2009 virtually all seasonal A(H1N1) viruses circulating worldwide were resistant to OS (93% global resistance) [72]**.** The initial rate at which NA H275Y OS-resistant seasonal A(H1N1) viruses were detected in Portugal (2007/2008) was very close to the average rate reported for Europe (24.2%). During this season, the proportion of OS-resistant viruses in Europe varied widely by country, ranging from 0.9% in Italy to 68% in Norway, with this latter representing the highest frequency of OS resistance reported globally [79]. NA H275Y OS-resistant viruses from 2007/2008 and 2008/2009 exhibited similar IC_{50} fold-change values that overall ranged from ≈160 to 615-fold. Also, no difference was observed in the IC_{50} fold-change of the resistant viruses from the same influenza season that exhibited different overall NA genetic backgrounds (4 different backgrounds; 2008/2009) and/or specific NA amino acid changes. This lack of difference was supported by the fact that none of the genetic differences occurred closely to either residue 275 or NA active site. Most IC_{50} fold-change values were below the reference range considered by the WHO AVWG for NA H275Y substitution in a seasonal N1 background (321 to 2597-fold) [80], and those located within the range were positioned at or near its lower limit. This is probably related with technical issues, associated with the use of a different fluorescence reader and/or a

different assay protocol compared to those used in the studies on which these reference values were based (inter-laboratory variability). Supporting this are the lower IC_{50} fold-change estimates also reported in other national NAI susceptibility surveillance studies (from ≈260-fold on) [31,81,82]. All NA H275Y OS-resistant seasonal A(H1N1) viruses circulating in Portugal belonged to the genetic clade 2B and specifically to the Northern European lineage, as evidenced by the presence of the NA D354G reversion amino acid substitution. The clustering of all resistant viruses in an evolutionary group defined by the NA H275Y mutation confirmed that none, particularly those from 2007/2008, emerged locally as a result of drug use. The more extensive phylogenetic analysis performed with seasonal N1 NA genes, including a large number of additional sequences from worldwide NA H275Y variant viruses, suggested the occurrence of three different introductions for the OS-resistant viruses circulating in Portugal during each influenza season. This was evidenced by the clear separation of the OS-resistant viruses from either 2007/2008 or 2008/2009 in three evolutionary subgroups, with the viruses from each subgroup being closely related to other resistant viruses from different worldwide regions. The emergence of a fit and transmissible NA H275Y OS-resistant seasonal A(H1N1) variant has been explained by the presence of additional NA amino acid substitutions that compensated for the detrimental effect of H275Y substitution on virus fitness [83]. This compensatory role is currently attributed to NA R222Q, V234M, D344N and D354G amino acid substitutions [48], which were identified in all NA H275Y OS-resistant viruses characterized genotypically. It is however still puzzling why NA H275Y A(H1N1) variant viruses spread over their OS-sensitive counterparts, outcompeting them from circulation, even more in a context of overall low drug use. Two evolutionary mechanisms have been proposed, specifically: (1) HA-NA functional balance (better balance between HA receptor-binding and NA receptor-destroying activities) [36,79,84-86]; and (2) genetic hitchhiking (physical linkage of NA H275Y substitution to beneficial amino acid substitutions) [37,87,88]. But, both mechanisms involve the potential fitness-compensatory substitutions above referred or the HA A189T amino acid substitution, all also characteristic of the clade 2C OS-resistant viruses that did not spread worldwide [37,87]. As evidenced in this study, the HA A189T substitution implied an amino acid change in the RBS of the protein that is the major determinant of both receptor-binding affinity and specificity [89]. Also, it was the only amino acid substitution differentiating the latter clade 2B OS-resistant viruses that fixed NA H275Y in A(H1N1) virus population (2008/2009 viruses) from those initially circulating (2007/2008 viruses)(NA and HA

genes). Considering that amino acid substitutions in PB1-F2 and PB2 were found to distinguish clade 2B and 2C OS-resistant viruses [36,87], it is possible that genetic hitchhiking may have in alternative involved other genetic changes located elsewhere in the viral genome, particularly in the polymerase complex genes [37,87]. It has been in fact suggested that the key to completely understand why NA H275Y OS-resistant seasonal A(H1N1) viruses became dominant is to look for the virus as a whole and to investigate all other segments of influenza virus genome [34,35,90]. This will be addressed in the next chapter of this PhD thesis (genome-wide study; Chapter 5). HA D186G and G152E amino acid substitutions were found in, respectively, a single and two NA H275Y OS-resistant seasonal A(H1N1) viruses from 2007/2008 and 2008/2009. Besides the structural evidence that both substitutions may affect NAI susceptibility (location at or near the RBS), residue 186 is know to play a key role in the effective binding to human-like α(2,6) receptors [91], while residue 152 is associated with *in vitro* selection of an influenza variant after serial passage under LAN drug pressure in 2009 pandemic A(H1N1) subtype (K153E variant; 2009 pandemic H1 numbering) [92].

The single NA H275Y OS-resistant A(H1N1)pdm09 virus detected exhibited an ≈250-fold decrease in OS susceptibility, which was within the reference range considered by the WHO AVWG for NA H275Y substitution in a 2009 pandemic N1 background (221 to 1637-fold) [80]. The virus also exhibited an amino acid substitution near the RBS in HA - N156D substitution, but probably as an artefact of cell culture. Several amino acid substitutions at residue 156 of H1 HA, including the one identified in this virus (N156D/S/K/T), have been described as commonly but not exclusively associated with virus propagation in cell culture [93,94]. NA H275Y substitution was also detected in the clinical specimen from another case of A(H1N1)pdm09 virus infection received in 2010/2011 with a high level of suspicion of clinical resistance to OS, but in only 26.2% of the virus population. The patient was also hospitalized with severe influenza illness but had no underlying risk condition. However, it was not possible to analyse this mixed population of H275Y/H virus through phenotypic assay, as not enough volume of clinical specimen was available for viral isolation. According to the WHO laboratory guidelines for interpretation of NA H275Y/H mixed virus populations, this case should be defined as ambiguous and not as resistant (H275Y variant ≥50%) [95]. Patient clinical data suggested, however, that this minor viral sub-population of NA H275Y OS-resistant viruses might have been sufficient to reduce treatment efficacy (clinical suspicion of resistance). Since

no information was available about the timing between the onset of symptoms and the beginning of antiviral therapy, which is critical for treatment efficacy (<48h) [96], it was decided to more accurately define the case as ambiguous. Mixed populations of NA H275Y/H A(H1N1)pdm09 viruses have been identified worldwide in either clinical specimens or virus isolates, creating additional challenges in the laboratory diagnosis of resistance [95,97]. The two NA H275Y A(H1N1)pdm09 variant viruses detected in Portugal emerged under drug-selective pressure (OS therapy), which is in agreement with the major role that drug use has been playing in the emergence of OS resistance in 2009 A(H1N1) pandemic subtype. Most NA H275Y OS-resistant A(H1N1)pdm09 viruses detected worldwide were recovered from patients undergoing OS therapy, many of whom also shared an immunocompromised condition [59].

4.2.3.2 Influenza Viruses with Decreased Susceptibility to Oseltamivir and/or Zanamivir

Decreased susceptibility to OS and/or ZA was detected in all influenza virus types and subtypes, in a limited number of viruses with no known association to drug use – natural decreased susceptibility. No predominance for decreased susceptibility to either OS or ZA was observed, with the total number of viruses exhibiting decreased susceptibility to OS (n=12; 2 seasonal A(H1N1), 3 A(H3N2), 3 B and 4 A(H1N1)pdm09), ZA (n=13; 1 A(H3N2), 8 B and 4 A(H1N1)pdm09), or to both NAIs (n=10; 1 A(H3N2), 2 B and 7 A(H1N1)pdm09) being very similar. Also, all viruses exhibited only a slight decrease in susceptibility, sharing an upper outlier status and a NI phenotype. The decrease in susceptibility was predominantly ≤2-fold, extending to 3 and 4-fold in few exceptional cases. The increasing trend observed in the fold-change decrease in ZA susceptibility of influenza B upper outlier viruses over time, from ≤2-fold in 2004/2005 to ≈3-fold during 2006/2007 to 2008/2009 and then to ≈4-fold in both the pandemic period and 2010/2011, was most probably an artefact of the combined analysis of B/VIC and B/YAM-lineage viruses and/or the use of wide-ranging combined cut-offs (pandemic period – 2012/2013). Both were needed due to constraints in the number of influenza B viruses analysed, from either the non-predominant or both lineages.

The three influenza A viruses with an ≈4-fold decrease in OS and ZA (A/Portugal/43/2012, A(H3N2)) or just OS (A/Portugal/17/2009 and

A/Portugal/82/2009, A(H1N1)pdm09) susceptibility, contained the NA I222V amino acid substitution (N2 numbering; I223V in N1 numbering). This substitution is known to reduce OS susceptibility by 2 to 4-fold in A(H3N2) viruses and by 6-fold in A(H1N1)pdm09 viruses [80,98,99], explaining therefore the decreases observed. It is also known to reduce ZA susceptibility by 2-fold in A(H1N1)pdm09 viruses [80], which was precisely the decrease observed for both virus isolates. Its effect on ZA susceptibility is still unknown for A(H3N2) subtype, but the previous study of Baz and colleagues suggests no impact [99]. They identified the NA I222V substitution together with NA E119V amino acid substitution in an A(H3N2) virus with an ≈2-fold decreased susceptibility to ZA and it is known that E119V alone reduces the susceptibility by 1 to 7-fold [80]. The 4-fold decreased susceptibility of A/Portugal/43/2012 to ZA may therefore be a result of the presence of NA D251G amino acid substitution. This substitution was previously reported in an A(H3N2) virus with an ≈5-fold decrease in ZA susceptibility [71], but it was not considered for the list of amino acid substitutions in NA causing decreased susceptibility (see Table 1.2, Literature Review), as its minor effect on susceptibility was not yet confirmed by reverse genetics (RG). This was the first time that both A(H3N2) and A(H1N1)pdm09 viruses carrying NA I222V substitution were reported in untreated patients. Previously, it had only been described in viruses recovered from patients undergoing OS therapy, in association with NA E119V (A(H3N2)) or H274Y (A(H1N1)pdm09; N2 numbering) substitution, or in reverse engineered viruses (A(H1N1)pdm09) [92,99-102]. Moreover, the two NA I222V A(H1N1)pdm09 variant viruses were probably linked through a transmission event, as evidenced by the 100% similarity of both NA and HA gene sequences and the close geographic and temporal relationship of the cases. NA I222V substitution is known to have no detrimental effect on the biological fitness of A(H1N1)pdm09 viruses [102].

The presence of NA D197N amino acid substitution in 44% of the virus population comprising the isolate B/Lisboa/34/2008 (D197N/D mixed virus population), explained the 3 and ≈2-fold decrease in, respectively, ZA and OS susceptibility, this latter only evidenced by chemiluminescent assay. NA D197N substitution is known to reduce ZA and OS susceptibility in influenza B viruses by, respectively, 2 to 17-fold and 4 to 10-fold, when present in the entire virus population [80]. The chemiluminescent assay revealed to be better at detecting the mixture of wild-type virus and D197N mutant virus with decreased susceptibility, which is in agreement with its higher sensitivity in measuring NA activity, compared to fluorescent MUNANA-based assays [103]. NA D197N variant has

been sporadically detected in circulating influenza B viruses with no or unknown association to drug use, particularly in China and Japan [48,104-108]. The B/YAM-lineage HA: B/VIC-lineage NA reassortant exhibiting a 2-fold decrease in OS susceptibility – virus isolate B/Lisboa/12/2005, carried the NA I240V amino acid substitution near the active site. The impact of this substitution on NAI susceptibility is unknown, but based on its location into the protein structure, it may play a role in the slight decrease observed. It is also possible that this decrease was just an artefact of the joint analysis of B/VIC and B/YAM-lineage viruses, as it was identified against statistical cut-offs based on essentially IC_{50} values of B/YAM-lineage viruses that over the 10-year period studied (2004-2013) tended to be lower than those of B/VIC lineage. Further RG studies will be important to clarify the effect of NA I240V substitution on influenza B virus NAI susceptibility. Phylogenetic analysis of influenza B NA and HA genes revealed to be essential to identify B/Lisboa/12/2005 as an inter-lineage reassortant and to accurately interpret IC_{50} data.

All other influenza A and B viruses exhibiting decreased susceptibility to OS and/or ZA (≤3-fold influenza A; ≤4-fold influenza B) contained no specific amino acid substitution in NA or only specific substitutions located away from the NA active site, which are unlikely to affect virus susceptibility. This lack of genetic evidence suggests that ≤3-fold and ≤4-fold decreases in phenotypic susceptibility can be an inherent characteristic of, respectively, influenza A and B viruses. It is therefore questionable whether it would be more appropriate to consider these viruses as sensitive, particularly those containing no specific NA amino acid substitution.

The A(H3N2) virus isolate A/Portugal/29/2012 with a 2-fold decreased susceptibility to OS contained an amino acid substitution at the HA RBS - T135A substitution. Moreover, it is known that the amino acid change to alanine (A) at residue 135 in seasonal H1 HA (G135A; G132A in H1 numbering) reduces the virus's binding affinity for cellular receptors, having emerged in a reassortant virus lacking sialidase activity (compensatory change for the loss of viral sialidase) [109]. Since HA amino acid changes with these characteristics are expected to reduce NAI susceptibility [110], it is possible that this A(H3N2) virus isolate presents in fact a different susceptibility to NAIs than that detected *in vitro* through NA inhibition assay (NA-mediated susceptibility). Further *in vitro* antiviral susceptibility testing accounting for HA-mediated changes is required to clarify the effect of H3 HA T135A substitution. HA N129D, K153E and N156K amino acid substitutions near the RBS were detected in the A(H1N1)pdm09 virus isolate A/Portugal/07/2011 with an ≈2-fold decreased susceptibility to both OS and ZA. All

three substitutions have been reported among circulating A(H1N1)pdm09 viruses, particularly the two latter ones [29,111,112]. HA K153E substitution is known to be cell culture-selected [29] and its presence as a mixed virus population (K153E/K) supports this association to cell culture. Amino acid substitutions at residue 156 have also been associated with virus propagation in cell culture, as above-mentioned for NA H275Y OS-resistant A(H1N1)pdm09 virus, but N156K substitution was found more commonly in clinical specimens than in cell culture isolates [29]. Also, HA N156K may play a role in enhancing viral fitness, as evidenced in a previous *in vivo* study conducted in ferrets, in which the HA N156K mutant virus showed to be fitter than the wild-type counterpart (potential fitness-enhancing mutation) [29]. The authors suggested that the N156K substitution increases the receptor-binding affinity of virus HA, based on the altered receptor-binding preferences of the mutant virus and on its computationally predicted effect on HA binding, stability and receptor specificity. All other influenza viruses exhibiting decreased susceptibility contained no specific HA amino acid substitutions or only specific substitutions located away from the RBS.

4.2.3.3 Drug-Sensitive Viruses and Relevant Amino Acid Changes

All influenza viruses with known association to antiviral use except the NA H275Y OS-resistant A(H1N1)pdm09 virus A/Portugal/03/2011, were sensitive to OS and/or ZA. This included the A(H1N1)pdm09 virus isolate A/Portugal/28/2009, recovered from a patient suspected of having developed drug-resistant influenza. An alternative explanation for the reduced treatment efficacy observed may be a delay >48h between the onset of symptoms and the beginning of antiviral therapy (starting date not available) that is known to compromise drug efficacy [96].

The increased susceptibility to OS and/or ZA exhibited by several influenza viruses from all different types and subtypes (phenotypic lower outlier viruses) appear to be an inherent characteristic of these viruses, as no genetic evidence for the increase observed was found. The viruses contained either no specific amino acid substitution in NA (majority) or only specific substitutions located far away from the active site.

Both D151N and Y155H amino acid substitutions in N2 and 2009 pandemic N1 NA, respectively, revealed to have no effect on NAI susceptibility. NA D151N substitution was detected as a mixed virus population in an A(H3N2) virus sensitive to both OS and ZA. Its presence as viral quasispecies supports the association to virus culturing that has been evidenced in several previous studies [31,113,114]. McKimm-Breschkin and colleagues had previously described the effect of NA D151N on NAI susceptibility but the results were unclear [115]. Two of the D151N A(H3N2) variant viruses were sensitive to both NAIs, while a third one exhibited an ≈7-fold decreased susceptibility to OS (fluorescent assay data). The results here obtained support the lack of effect on NAI susceptibility, but it will be important to confirm this phenotype in more specific studies, using RG. Amino acid substitutions at residue 151 in N2 NA are usually associated with high decreases in NAI susceptibility, conferring RI by OS (D151E) or (H)RI by ZA (D151A/G/V) [80]. NA Y155H substitution was detected in several A(H1N1)pdm09 viruses sensitive to both NAIs. This substitution conferred (H)RI by NAIs in former circulating A(H1N1) viruses but its effect in A(H1N1)pdm09 viruses was still unknown. These results were the first evidence that NA Y155H substitution has no effect on the NAI susceptibility of current circulating 2009 pandemic A(H1N1) viruses, as timely reported in Giria *et al.* [116]. Similar results were recently obtained in an influenza antiviral susceptibility surveillance study carried out in Spain, confirming the lack of effect of NA Y155H [117]. Structural differences between seasonal and 2009 pandemic N1 NA may explain the virus-specific nature of the effect of this substitution on susceptibility. The lack of the 150-cavity characteristic of group 1 NAs in 2009 pandemic N1 NA is the main feature distinguishing the structure of these two N1 NAs. But, it probably does not explain the difference observed, as avian influenza A(H5N1) viruses carrying NA Y155H substitution were also sensitive to NAIs and the 150-cavity is present in their NA [98].

The R142G and S262N amino acid substitutions in H3 HA known to occur clinically under OS drug-selective pressure [118,119], were detected in single sensitive viruses with, respectively, known and unknown association to drug use. The D222G amino acid substitution in 2009 pandemic H1 HA was also detected in a single virus sensitive to both NAIs in NA inhibition assay. This amino acid substitution is known to cause a high decrease (≤100-fold) in the NAI susceptibility of former circulating A(H1N1) viruses (D221G; seasonal H1 numbering) [49], but in 2009 pandemic A(H1N1) subtype it was only selected *in vitro* after serial passages under drug-selective pressure (LAN) [92]. Further *in*

vitro susceptibility testing is essential to determine how these three HA amino acid substitutions affect NAI susceptibility in the specific influenza virus subtypes on which were detected (HA-mediated susceptibility). Based on both epidemiological and clinical data, it is not, however, expected a significant impact for HA D222G substitution. HA D222G variant has been detected at low to medium-low rates (5% to 24%) in A(H1N1)pdm09 viruses recovered from patients with severe disease and admitted in ICU facilities [120,121]. Since the use of NAIs is highly recommended for such patients, these were most probably under antiviral therapy and no report of suspected clinical resistance was found in the literature.

4.2.3.4 Natural In Vitro Susceptibility of Circulating Viruses

The natural *in vitro* susceptibility of human influenza viruses circulating in Portugal from 2004/2005 to 2012/2013 to NAIs varied over time, but with no particular trend and in essentially different ways to OS and ZA. Given the very low prescription of NAIs in Portugal (National Influenza Surveillance Programme data; 2005/2006 to 2008/2009), the variations in the baseline phenotypic drug susceptibility were unlikely to be related to drug use, reflecting most probably natural evolutionary changes in influenza virus NA.

The marked decrease in the natural *in vitro* susceptibility of circulating seasonal A(H1N1) viruses to OS between 2007/2008 and 2008/2009 was driven by the worldwide emergence of a NA H275Y OS-resistant variant that, as above-mentioned, resulted in an overall global resistance to OS. The slight (≤2-fold) but significant change in their natural susceptibility to both NAIs between 2005/2006 and 2007/2008 also matched the evolutionary drift from NC/99-like to BR/07-like viruses. The susceptibility changed, however, in opposite ways for the two NAIs, decreasing for OS and increasing for ZA. Previous studies showed that the NA of BR/07-like viruses had a higher affinity for sialic acid (natural substrate) and OS and ZA, compared to NC/99-like viruses [35,82,84], supporting the role of evolutionary drift in the increased natural susceptibility of the viruses to ZA. This role may have been played by NA R222Q and/or D344N amino acid substitutions (D344N only not present in one reference BR/07-like virus) that are known to significantly increase NA binding affinity for the substrate (fitness-compensatory role in NA H275Y variant) [34-37]. All other NA amino acid substitutions characteristic of BR/07-

like viruses except G249K are located distantly from the active site and thereby are unlikely to affect NA binding affinity. The lower natural susceptibility of BR/07-like viruses to OS is puzzling and difficult to understand as it goes against the known higher affinity of the virus NA for drug binding.

The change to a new drift variant may also explain the 2-fold decrease in the natural *in vitro* susceptibility of circulating A(H3N2) viruses to OS and ZA between 2008/2009 and 2011/2012. A(H3N2) viruses from 2011/2012 onwards belonged to the Victoria/208 genetic clade, while those circulating previously belonged to either Brisbane/10, Wisconsin/67 or California/07 genetic clades. Based on its occurrence near the active site, both NA amino acid substitutions characterizing A/Victoria/208/2009-like viruses (S367N and K369T) could have played a role in this decrease.

The slightly lower natural *in vitro* susceptibility of influenza B viruses circulating during the pandemic period to OS (2 to 3-fold) and of those circulating during 2005/2006 to ZA (2 to ≈4-fold) were probably artefacts of, respectively, the use of wide-ranging combined cut-offs and joint analysis of B/VIC and B/YAM-lineage viruses. The potential role that influenza B lineage plays in NAI susceptibility, particularly to ZA, evidenced by overall comparative analysis of the non-outlier IC_{50} values of B/VIC and B/YAM-lineage viruses, supports this latter. A decrease in influenza B virus natural susceptibility to NAIs, less evident for OS, was also observed between 2004/2005 and 2005/2006 in a previous susceptibility surveillance study covering the period from 1998/1999 to 2005/2006 [122]. However, the authors attributed the decrease to the evolutionary drift in NA and not to virus lineage, considering that the NAs of the B/VIC-lineage viruses circulating in 2005/2006 were still from the B/YAM lineage (origin in a B/VIC-lineage HA: B/YAM-lineage NA reassortant from 2001/2002). Two of the NA amino acid substitutions here identified as specific of B/VIC-lineage viruses - NA K219N and K373E, were also reported in this previous study for the B/VIC-lineage viruses from 2005/2006. Both substitutions occurred near the active site and thereby may have played a role in the decrease observed. Large-scale *in vitro* NAI susceptibility testing of B/VIC and B/YAM-lineage viruses is essential to clarify the effect of influenza B lineage on NAI susceptibility, even more when the recent worldwide study of Okomo-Adhiambo and colleagues suggested its impact on exclusively OS susceptibility (no impact on ZA, PER and LAN susceptibility) [73].

No genetic evidence in NA was found for the slight decrease in the natural *in vitro* susceptibility of A(H1N1)pdm09 viruses to OS between the pandemic period and 2010/2011. Only the NA N369K amino acid substitution that is known to significantly increase NA binding affinity for the substrate (fitness-compensatory role in NA H275Y variant) [42], distinguished the A(H1N1)pdm09 viruses from these two time periods. If affecting NAI susceptibility, this substitution would increase and not decrease the virus natural susceptibility to the drug. It is important to note that this decrease in susceptibility occurred after the higher use of OS during 2009 influenza pandemic [123].

Further RG studies will be essential to confirm the role of seasonal N1 NA R222Q and D344N, N2 NA S367N and K369T, and influenza B NA K219N and K373E amino acid substitutions in NAI susceptibility.

Both former and current circulating A(H1N1) viruses, as well as influenza B viruses, were more susceptible to ZA than to OS, while the inverse was observed in A(H3N2) subtype. These drug-specific differences have been reported in other worldwide [98,115,124] and national NAI susceptibility surveillance studies [67,68,125-128], and are known to be related to differences in the chemical structure of the drugs [129]. OS and ZA have different core ring structures and active moieties, binding differently at the enzyme catalytic site [36,130]. Drug-specific differences up to 5-fold were here observed in type B influenza, while those in influenza A subtypes varied up to 3-fold except in seasonal A(H1N1) viruses (≤2-fold).

Influenza virus natural susceptibility to OS and ZA also differed according to the virus type and subtype, in a similar way to both drugs. The only exception were the A(H3N2) and A(H1N1)pdm09 viruses that exhibited a similar overall susceptibility to ZA. Overall, the differences observed were ordered as follows: B **<** seasonal A(H1N1) **<** A(H1N1)pdm09 **<**$_{\text{oseltamivir}}$/**~**$_{\text{zanamivir}}$ A(H3N2). The same order has been reported for OS in other worldwide [71,115,124] and national NAI susceptibility surveillance studies [67,126-128,131]. But, in all of them except one [67], A(H3N2) viruses were less susceptible to ZA than seasonal A(H1N1) viruses. Influenza type and subtype-specific differences are likely to be related to differences in the structure of the virus NAs that can result in different binding affinities for the drug [125]. Based on the lesser therapeutic response to OS observed in patients with influenza B illness compared to those with influenza A [72,132,133], it has been

suggested that the lower *in vitro* NAI susceptibility naturally exhibited by influenza B viruses might have a slight impact in the clinic.

4.3 CONCLUSIONS

This study marked the beginning of influenza antiviral susceptibility testing and monitoring activities in Portugal. As the activities were extended over a wide time period (10 years; 2004-2013), it also generated comprehensive information on the susceptibility of circulating human influenza viruses to the three antivirals approved nationally for clinical use (AMA, OS, and ZA). This information contributed to the global and European influenza surveillance on antiviral susceptibility and allowed to improve the knowledge on the relationship between virus NAI susceptibility phenotype and genotype, which is one of the major current challenges in the field, and on the natural variations in the *in vitro* susceptibility of circulating viruses over time. In a broader-sense, this study also provided a better understanding of the evolutionary dynamics of influenza virus NA and HA genes.

BOX 4.2 - MAIN FINDINGS

Specific objective 1(b)

M2 inhibitor resistance

- M2 inhibitor resistance was detected in A(H3N2) subtype since 2005/2006 (S31N, except 3 S31N/V27A double mutants) and in A(H1N1)pdm09 subtype since its emergence (S31N; naturally-occurring resistance).
- The frequency of A(H3N2) drug-resistant viruses varied initially between 100% and 74.5%, settling at 100% from 2008/2009 onwards.

OS and ZA susceptibility

- Virtually all human influenza viruses circulating in Portugal from 2004/2005 to 2012/2013, except those from seasonal A(H1N1) subtype, were sensitive to OS and ZA.
- OS resistance in seasonal A(H1N1) subtype increased from 20.7% in 2007/2008 to 100% in 2008/2009. All cases were associated with the worldwide spread of a fit and transmissible NA H275Y OS-resistant variant.
- NA H275Y OS-resistant viruses belonged to the genetic clade 2B (Northern European lineage) and apparently resulted from three different introductions of the NA H275Y variant in Portugal during 2007/2008 and 2008/2009.
- OS resistance in A(H1N1)pdm09 subtype was detected in a single virus from 2010/2011 recovered from an immunocompromised and pregnant patient under antiviral therapy (suspected case of clinical resistance).

BOX 4.2 - MAIN FINDINGS (cont.)

- A patient clinically suspected of having developed drug-resistant influenza harboured a minor viral sub-population of NA H275Y OS-resistant A(H1N1)pdm09 virus (<30%).
- Few influenza viruses from all different types and subtypes exhibited a natural ≤2 to 4-fold decreased susceptibility to OS and/or ZA (NI phenotype; no known association to drug use).
- NA I222V amino acid substitution (N2 numbering) conferred the ≈4-fold decrease in OS susceptibility observed in a single A(H3N2) virus and in two A(H1N1)pdm09 viruses. It also conferred the 2-fold decreased susceptibility to ZA exhibited by both A(H1N1)pdm09 viruses.
- NA I222V A(H3N2) and A(H1N1)pdm09 variants were for the first time recovered from untreated patients.
- NA D251G amino acid substitution may confer a 4-fold decreased susceptibility to ZA in A(H3N2) subtype (novel amino acid change).
- A sub-population of NA D197N influenza B virus (44%) exhibited a 3 and ≈2-fold decreased susceptibility to ZA and OS, respectively.
- NA D151N (N2 numbering) and NA Y155H (N1 numbering) amino acid substitutions have no effect on NAI susceptibility in, respectively, A(H3N2) and A(H1N1)pdm09 subtypes.
- An A(H1N1)pdm09 virus with ≈2-fold decreased susceptibility to OS and ZA harboured the potential fitness-enhancing HA N156K mutation.

Specific objective 1(c)

- Fold decreases ≤3 and ≤4 in *in vitro* NAI susceptibility (phenotype) can be an inherent characteristic of, respectively, influenza A and B viruses, having no amino acid genetic change underlying it (genotype).

Specific objective 1(d)

- Natural *in vitro* NAI susceptibility of circulating human influenza viruses varied over time but with no particular trend and in essentially different ways to OS and ZA.
- Marked decrease in the natural *in vitro* susceptibility of seasonal A(H1N1) viruses to OS between 2007/2008 and 2008/2009 as a result of the worldwide spread of a fit and transmissible NA H275Y OS-resistant variant (only drug-resistant viruses circulated in 2008/2009 – IC_{50} outlier median).
- Evolutionary drift to A/Brisbane/59/2007-like viruses in seasonal A(H1N1) subtype and to A/Victoria/208/2009-like viruses in A(H3N2) subtype might have had a minor (≤2-fold change) but significant impact on virus natural susceptibility to OS and/or ZA.
- Influenza B lineage may play a role in virus susceptibility to OS and particularly ZA.
- Natural *in vitro* susceptibility of A(H1N1)pdm09 viruses to OS decreased slightly (<2-fold) after the higher use of the drug during 2009 influenza pandemic but no genetic support was found.

Complementary study on influenza antiviral prescription

- Antivirals were rarely prescribed to patients presenting with ILI to healthcare systems covered by the National Influenza Surveillance Programme in Portugal from 2005/2006 to 2008/2009, reflecting the national public health policy for antiviral therapy in seasonal influenza that, at that time, was essentially restricted to patients with underlying risk conditions.

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SUPPLEMENTARY DATA

Table S4.1 List of the accession number of the hemagglutinin and neuraminidase nucleotide sequences of influenza viruses circulating in Portugal used in this study and shared in public-access databases.

Influenza virus type/subtype	Influenza season/ Pandemic period[a]	Sequence database	Accession number	Segment	Virus isolate
A(H3N2)	2004/2005	NCBI Influenza Virus Resource	EU128342	HA	A/Lisboa/04/2004
			EU128343	HA	A/Lisboa/05/2004
			EU128344	HA	A/Lisboa/06/2005
			EU128346	HA	A/Lisboa/10/2005
			EU128345	HA	A/Lisboa/12/2005
			EU128347	HA	A/Lisboa/15/2005
			EU128348	HA	A/Lisboa/23/2005
			EU128350	HA	A/Lisboa/33/2005
			EU128351	HA	A/Lisboa/34/2005
			EU128352	HA	A/Lisboa/40/2005
			EU128353	HA	A/Lisboa/41/2005
			EU128354	HA	A/Lisboa/50/2005
			EU128355	HA	A/Lisboa/51/2005
			EU128357	HA	A/Lisboa/53/2005
			EU128358	HA	A/Lisboa/82/2005
			EU128359	HA	A/Lisboa/87/2005
			EU128361	HA	A/Lisboa/90/2005
			EU128362	HA	A/Lisboa/91/2005
B	2004/2005	NCBI Influenza Virus Resource	EU370378	HA	B/Lisboa/01/2004
			EU370381	HA	B/Lisboa/03/2005
			EU370386	HA	B/Lisboa/11/2005
			EU370387	HA	B/Lisboa/12/2005
			EU370388	HA	B/Lisboa/13/2005
			EU370389	HA	B/Lisboa/19/2005
			EU370390	HA	B/Lisboa/22/2005
			EU370379	HA	B/Lisboa/32/2005
A(H1N1)pdm09	Pandemic period	GISAID EPIFLU™	EPI500665	HA	A/Portugal/01/2009
			EPI500664	NA	A/Portugal/01/2009
			EPI500667	HA	A/Portugal/02/2009
			EPI500666	NA	A/Portugal/02/2009
			EPI500669	HA	A/Portugal/03/2009
			EPI500668	NA	A/Portugal/03/2009
			EPI500671	HA	A/Portugal/04/2009
			EPI500670	NA	A/Portugal/04/2009
			EPI500673	HA	A/Portugal/05/2009
			EPI500672	NA	A/Portugal/05/2009
			EPI500675	HA	A/Portugal/06/2009
			EPI500674	NA	A/Portugal/06/2009
			EPI500677	HA	A/Portugal/07/2009
			EPI500676	NA	A/Portugal/07/2009
			EPI500679	HA	A/Portugal/08/2009
			EPI500678	NA	A/Portugal/08/2009
			EPI500681	HA	A/Portugal/09/2009
			EPI500680	NA	A/Portugal/09/2009
			EPI500683	HA	A/Portugal/10/2009
			EPI500682	NA	A/Portugal/10/2009
			EPI500685	HA	A/Portugal/11/2009
			EPI500684	NA	A/Portugal/11/2009
			EPI500687	HA	A/Portugal/12/2009
			EPI500686	NA	A/Portugal/12/2009
			EPI500689	HA	A/Portugal/13/2009
			EPI500688	NA	A/Portugal/13/2009
			EPI500691	HA	A/Portugal/14/2009
			EPI500690	NA	A/Portugal/14/2009
			EPI500693	HA	A/Portugal/15/2009
			EPI500692	NA	A/Portugal/15/2009
			EPI500696	HA	A/Portugal/17/2009
			EPI500695	NA	A/Portugal/17/2009
			EPI500698	HA	A/Portugal/18/2009
			EPI500697	NA	A/Portugal/18/2009
			EPI500700	HA	A/Portugal/19/2009
			EPI500699	NA	A/Portugal/19/2009
			EPI500702	HA	A/Portugal/23/2009
			EPI500701	NA	A/Portugal/23/2009

(Table S4.1 cont.)

Influenza virus type/subtype	Influenza season/ Pandemic period[a]	Sequence database	Accession number	Segment	Virus isolate
A(H1N1)pdm09	Pandemic period	GISAID EPIFLU™	EPI500704	HA	A/Portugal/25/2009
			EPI500703	NA	A/Portugal/25/2009
			EPI500706	HA	A/Portugal/26/2009
			EPI500705	NA	A/Portugal/26/2009
			EPI500708	HA	A/Portugal/27/2009
			EPI500707	NA	A/Portugal/27/2009
			EPI500710	HA	A/Portugal/28/2009
			EPI500709	NA	A/Portugal/28/2009
			EPI500712	HA	A/Portugal/30/2009
			EPI500711	NA	A/Portugal/30/2009
			EPI500714	HA	A/Portugal/31/2009
			EPI500713	NA	A/Portugal/31/2009
			EPI500716	HA	A/Portugal/33/2009
			EPI500715	NA	A/Portugal/33/2009
			EPI500718	HA	A/Portugal/41/2009
			EPI500717	NA	A/Portugal/41/2009
			EPI500720	HA	A/Portugal/45/2009
			EPI500719	NA	A/Portugal/45/2009
			EPI500722	HA	A/Portugal/46/2009
			EPI500721	NA	A/Portugal/46/2009
			EPI500724	HA	A/Portugal/47/2009
			EPI500723	NA	A/Portugal/47/2009
			EPI500727	HA	A/Portugal/52/2009
			EPI500726	NA	A/Portugal/52/2009
			EPI500729	HA	A/Portugal/53/2009
			EPI500728	NA	A/Portugal/53/2009
			EPI500731	HA	A/Portugal/63/2009
			EPI500730	NA	A/Portugal/63/2009
			EPI500733	HA	A/Portugal/64/2009
			EPI500732	NA	A/Portugal/64/2009
			EPI500735	HA	A/Portugal/66/2009
			EPI500734	NA	A/Portugal/66/2009
			EPI500737	HA	A/Portugal/77/2009
			EPI500736	NA	A/Portugal/77/2009
			EPI500739	HA	A/Portugal/78/2009
			EPI500738	NA	A/Portugal/78/2009
			EPI500741	HA	A/Portugal/80/2009
			EPI500740	NA	A/Portugal/80/2009
			EPI500743	HA	A/Portugal/82/2009
			EPI500742	NA	A/Portugal/82/2009
			EPI500745	HA	A/Portugal/90/2009
			EPI500744	NA	A/Portugal/90/2009
			EPI500747	HA	A/Portugal/101/2009
			EPI500746	NA	A/Portugal/101/2009
			EPI500749	HA	A/Portugal/114/2009
			EPI500748	NA	A/Portugal/114/2009
			EPI500752	HA	A/Portugal/01/2010
			EPI500751	NA	A/Portugal/01/2010
			EPI500755	HA	A/Portugal/02/2010
			EPI500754	NA	A/Portugal/02/2010
	2010/2011	GISAID EPIFLU™	EPI500757	HA	A/Portugal/03/2011
			EPI500756	NA	A/Portugal/03/2011
			EPI500759	HA	A/Portugal/07/2011
			EPI500758	NA	A/Portugal/07/2011
			EPI500761	HA	A/Portugal/10/2011
			EPI500760	NA	A/Portugal/10/2011
			EPI500764	HA	A/Portugal/21/2011
			EPI500763	NA	A/Portugal/21/2011
			EPI500766	HA	A/Portugal/22/2011
			EPI500765	NA	A/Portugal/22/2011
			EPI500769	HA	A/Portugal/28/2011
			EPI500768	NA	A/Portugal/28/2011

[a] 2009 pandemic period – from 11th June 2009 to 9th August 2010; NA: Neuraminidase; HA: Hemagglutinin

NCBI Influenza Virus Resource database: http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html

GISAID EpiFlu™ database: http://platform.gisaid.org.

The sequences were either generated in this study or were already available at the laboratory sequence database, having been obtained in the context of the study on the evolutionary dynamics of the two major antigens of influenza viruses.

I acknowledge the authors, originating and submitting laboratories of the HA and NA nucleotide sequences of worldwide reference influenza viruses retrieved from GISAID EpiFlu™ and NCBI Influenza Virus Resource databases that were used in this study.

Table S4.2 Detailed information on the hemagglutinin and neuraminidase nucleotide sequences of worldwide reference influenza viruses used in this study, retrieved from GISAID EpiFlu™ and NCBI Influenza Virus Resource databases.

Influenza (sub)type	Accession number	Segment	Country	Collection date (year-month-day)	Reference virus	Originating Lab	Submitting Lab	Authors
Former seasonal A(H1N1)	EPI249530	HA	Australia	2007-Jan-01	***A/Brisbane/59/2007***	-	Other Database Import	Webby,R.; Webster,R.G.; Govorkova,E.; Duan,S.
	EPI249532	NA	Australia	2007-Jan-01	***A/Brisbane/59/2007***	-	Other Database Import	Webby,R.; Webster,R.G.; Govorkova,E.; Duan,S.
	EPI58144	HA	Chile	1983-Jan-01	***A/Chile/01/1983***	-	Other Database Import	-
	EPI58149	NA	Chile	1983-Jan-01	***A/Chile/01/1983***	-	Other Database Import	-
	EPI357133	HA	Egypt	2007-Oct-31	A/Egypt/10/2007	VACSERA	National Institute for Medical Research	-
	EPI357134	NA	Egypt	2007-Oct-31	A/Egypt/10/2007	VACSERA	National Institute for Medical Research	-
	EPI509645	HA	United Kingdom	2004-May-20	A/England/40/2004	Health Protection Agency	National Institute for Medical Research	-
	EPI509646	NA	United Kingdom	2004-May-20	A/England/40/2004	Health Protection Agency	National Institute for Medical Research	-
	EPI141301	NA	United Kingdom	2007-Nov-15	A/England/557/2007	-	Other Database Import	-
	EPI168135	HA	United Kingdom	2007-Nov-15	A/England/557/2007	-	Other Database Import	-
	EPI155237	HA	Japan	2006-Jan-17	A/Fukushima/141/2006	-	Centers for Disease Control and Prevention	-
	EPI155235	NA	Japan	2006-Jan-17	A/Fukushima/141/2006	-	Centers for Disease Control and Prevention	-
	EPI211553	HA	Hong Kong (SAR)	2008-Jul-24	A/Hong Kong/1856/2008	Government Virus Unit	National Institute for Medical Research	-
	EPI211567	NA	Hong Kong (SAR)	2008-Jul-24	A/Hong Kong/1856/2008	Government Virus Unit	National Institute for Medical Research	-
	EPI509649	HA	Hong Kong (SAR)	2004-Jun-24	A/Hong Kong/2637/2004	Government Virus Unit	National Institute for Medical Research	-
	EPI116196	NA	China	2004-Jan-01	A/Hong Kong/2637/2004	-	Other Database Import	-
	EPI509368	HA	Hong Kong (SAR)	2006-Jul-17	A/Hong Kong/2652/2006	Government Virus Unit	National Institute for Medical Research	-
	EPI509369	NA	Hong Kong (SAR)	2006-Jul-17	A/Hong Kong/2652/2006	Government Virus Unit	National Institute for Medical Research	-
	EPI509650	HA	Hong Kong (SAR)	2004-Jun-29	A/Hong Kong/2765/2004	Government Virus Unit	National Institute for Medical Research	-
	EPI509651	NA	Hong Kong (SAR)	2004-Jun-29	A/Hong Kong/2765/2004	Government Virus Unit	National Institute for Medical Research	-
	EPI229176	HA	Hong Kong (SAR)	2008-Nov-24	A/Hong Kong/3192/2008	Government Virus Unit	National Institute for Medical Research	-
	EPI229175	NA	Hong Kong (SAR)	2008-Nov-24	A/Hong Kong/3192/2008	Government Virus Unit	National Institute for Medical Research	-
	EPI211552	HA	Netherlands	2007-Oct-12	A/Netherlands/345/2007	Erasmus University of Rotterdam	National Institute for Medical Research	-
	EPI211551	NA	Netherlands	2007-Oct-12	A/Netherlands/345/2007	Erasmus University of Rotterdam	National Institute for Medical Research	-
	EPI159392	HA	New Caledonia	1999-Jan-01	***A/New Caledonia/20/1999***	-	Other Database Import	-
	EPI159394	NA	New Caledonia	1999-Jan-01	***A/New Caledonia/20/1999***	-	Other Database Import	-
	EPI357156	HA	Norway	2007-Dec-08	A/Norway/1735/2007	Norwegian Institute of Public Health	National Institute for Medical Research	-
	EPI357157	NA	Norway	2007-Dec-08	A/Norway/1735/2007	Norwegian Institute of Public Health	National Institute for Medical Research	-
	EPI509308	HA	France	2006-Nov-30	A/Paris/160/2007	Institut Pasteur	National Institute for Medical Research	-
	EPI509309	NA	France	2006-Nov-30	A/Paris/160/2007	Institut Pasteur	National Institute for Medical Research	-
	EPI211539	HA	Seychelles	2008-Mar-06	A/Seychelles/2239/2008	Institut Pasteur de Madagascar	National Institute for Medical Research	-
	EPI211540	NA	Seychelles	2008-Mar-06	A/Seychelles/2239/2008	Institut Pasteur de Madagascar	National Institute for Medical Research	-
	EPI58239	HA	Singapore	1986-Jan-01	***A/Singapore/06/1986***	-	Other Database Import	-
	EPI58244	NA	Singapore	1986-Jan-01	***A/Singapore/06/1986***	-	Other Database Import	-
	EPI123778	HA	Solomon Islands	2006-Aug-21	***A/Solomon Islands/03/2006***	-	Other Database Import	-
	EPI123696	NA	Solomon Islands	2006-Aug-21	***A/Solomon Islands/03/2006***	-	Other Database Import	-
	EPI168229	HA	Russian Federation	2008-Feb-13	A/St. Petersburg/12/2008	-	Centers for Disease Control and Prevention	-
	EPI172726	NA	Russian Federation	2008-Feb-13	A/St. Petersburg/12/2008	-	Centers for Disease Control and Prevention	-
	EPI117358	HA	Australia	2005-Oct-07	A/Sydney/550/2005	-	Other Database Import	-
	EPI105054	HA	Taiwan	1986-Jan-01	A/Taiwan/01/1986	-	Other Database Import	-
	EPI105058	NA	Taiwan	1986-Jan-01	A/Taiwan/01/1986	-	Other Database Import	-
	EPI347370	HA	Greece	2005-Feb-03	A/Thessaloniki/24/2005	Aristotelian University of Thessaloniki	National Institute for Medical Research	-
	EPI166680	NA	Greece	2005-Feb-03	A/Thessaloniki/24/2005	-	Other Database Import	-
	EPI230610	HA	Russian Federation	1977-Jan-01	***A/USSR/90/1977***	-	Other Database Import	NIAID Influenza Genome Sequencing Consortium
	EPI230612	NA	Russian Federation	1977-Jan-01	***A/USSR/90/1977***	-	Other Database Import	NIAID Influenza Genome Sequencing Consortium

(Table S4.2 cont.)

Influenza (sub)type	Accession number	Segment	Country	Collection date (year-month-day)	Reference virus	Originating Lab	Submitting Lab	Authors
A(H3N2)	EPI349374	HA	United States	2011-Dec-07	A/Alabama/04/2011	ADPH Bureau of Clinical Laboratories	Centers for Disease Control and Prevention	-
	EPI349373	NA	United States	2011-Dec-07	A/Alabama/04/2011	ADPH Bureau of Clinical Laboratories	Centers for Disease Control and Prevention	-
	EPI278805	HA	United States	2010-Jul-13	A/Alabama/05/2010	U.S. Air Force School of Aerospace Medicine	Centers for Disease Control and Prevention	-
	EPI278804	NA	United States	2010-Jul-13	A/Alabama/05/2010	U.S. Air Force School of Aerospace Medicine	Centers for Disease Control and Prevention	-
	EPI365898	HA	China	1992-Jan-01	***A/Beijing/32/1992***	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI365900	NA	China	1992-Jan-01	***A/Beijing/32/1992***	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI367190	HA	China	1989-Jan-01	***A/Beijing/353/1989***	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI367192	NA	China	1989-Jan-01	***A/Beijing/353/1989***	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI347409	HA	Germany	2006-Feb-06	A/Berlin/02/2006	Robert-Koch-Institute	National Institute for Medical Research	-
	EPI353304	HA	Australia	2007-Feb-06	***A/Brisbane/10/2007***	Queensland Health Scientific Services	WHO Collaborating Centre for Reference and Research on Influenza	Iannello,P; Komadina,N
	EPI353303	NA	Australia	2007-Feb-06	***A/Brisbane/10/2007***	Queensland Health Scientific Services	WHO Collaborating Centre for Reference and Research on Influenza	Iannello,P; Komadina,N
	EPI168615	HA	Australia	2008-Jun-23	A/Brisbane/24/2008	-	Centers for Disease Control and Prevention	-
	EPI168614	NA	Australia	2008-Jun-23	A/Brisbane/24/2008	-	Centers for Disease Control and Prevention	-
	EPI228963	HA	United States	2004-Jan-01	***A/California/07/2004***	-	Other Database Import	Bragstad,K.; Nielsen,L.P.; Fomsgaard,A.
	EPI228966	NA	United States	2004-Jan-01	***A/California/07/2004***	-	Other Database Import	Bragstad,K.; Nielsen,L.P.; Fomsgaard,A.
	EPI302283	HA	United States	2010-Dec-08	A/California/28/2010	California Department of Health Services	Centers for Disease Control and Prevention	-
	EPI302282	NA	United States	2010-Dec-08	A/California/28/2010	California Department of Health Services	Centers for Disease Control and Prevention	-
	EPI417021	HA	Senegal	2012-Oct-11	A/Dakar/15/2012	Institut Pasteur de Dakar	National Institute for Medical Research	-
	EPI417022	NA	Senegal	2012-Oct-11	A/Dakar/15/2012	Institut Pasteur de Dakar	National Institute for Medical Research	-
	EPI417237	HA	United States	2012-Nov-12	A/Delaware/15/2012	Delaware Public Health Lab	Centers for Disease Control and Prevention	-
	EPI417236	NA	United States	2012-Nov-12	A/Delaware/15/2012	Delaware Public Health Lab	Centers for Disease Control and Prevention	-
	EPI346607	HA	United Kingdom	2011-Nov-16	A/England/259/2011	Health Protection Agency	National Institute for Medical Research	-
	EPI346608	NA	United Kingdom	2011-Nov-16	A/England/259/2011	Health Protection Agency	National Institute for Medical Research	-
	EPI301362	HA	United Kingdom	2010-Nov-26	A/England/4820166/2010	Health Protection Agency	National Institute for Medical Research	-
	EPI301361	NA	United Kingdom	2010-Nov-26	A/England/4820166/2010	Health Protection Agency	National Institute for Medical Research	-
	EPI358866	HA	United Kingdom	2006-Nov-22	A/England/560/2006	-	Other Database Import	Galiano,M.; Johnson,B.F.; Myers,R.; Ellis,J.; Daniels,R.; *et al.*
	EPI315043	NA	United Kingdom	2006-Nov-22	A/England/560/2006	-	Other Database Import	Galiano,M.; Johnson,B.F.; Myers,R.; Ellis,J.; Daniels,R.; *et al.*
	EPI215006	HA	United Kingdom	2008-Dec-30	A/England/687/2008	Health Protection Agency	National Institute for Medical Research	-
	EPI215005	NA	United Kingdom	2008-Dec-30	A/England/687/2008	Health Protection Agency	National Institute for Medical Research	-
	EPI358784	HA	China	2002-Jan-01	***A/Fujian/411/2002***	-	Other Database Import	Galiano,M.; Johnson,B.F.; Myers,R.; Ellis,J.; Daniels,R.; *et al.*
	EPI358786	NA	China	2002-Jan-01	***A/Fujian/411/2002***	-	Other Database Import	Galiano,M.; Johnson,B.F.; Myers,R.; Ellis,J.; Daniels,R.; *et al.*
	EPI123249	HA	United States	2006-Dec-28	A/Hawaii/06/2006	-	Centers for Disease Control and Prevention	-
	EPI123136	NA	United States	2006-Dec-28	A/Hawaii/06/2006	-	Centers for Disease Control and Prevention	-
	EPI342130	HA	United States	2009-Mar-30	A/Hawaii/07/2009	State of Hawaii Department of Health	Centers for Disease Control and Prevention	-
	EPI342129	NA	United States	2009-Mar-30	A/Hawaii/07/2009	State of Hawaii Department of Health	Centers for Disease Control and Prevention	-
	EPI126805	HA	Japan	2005-Oct-24	A/Hiroshima/52/2005	-	Other Database Import	Krammer,F.; Grabherr,R.
	EPI126807	NA	Japan	2005-Oct-24	A/Hiroshima/52/2005	-	Other Database Import	Krammer,F.; Grabherr,R.
	EPI211537	HA	Hong Kong (SAR)	2009-Mar-24	A/Hong Kong/1952/2009	Government Virus Unit	National Institute for Medical Research	-
	EPI211538	NA	Hong Kong (SAR)	2009-Mar-24	A/Hong Kong/1952/2009	Government Virus Unit	National Institute for Medical Research	-
	EPI211541	HA	Hong Kong (SAR)	2009-Apr-04	A/Hong Kong/1985/2009	Government Virus Unit	National Institute for Medical Research	-
	EPI211542	NA	Hong Kong (SAR)	2009-Apr-04	A/Hong Kong/1985/2009	Government Virus Unit	National Institute for Medical Research	-
	EPI353512	HA	Hong Kong (SAR)	2009-Apr-07	A/Hong Kong/2000/2009	Government Virus Unit	Centers for Disease Control and Prevention	-
	EPI353511	NA	Hong Kong (SAR)	2009-Apr-07	A/Hong Kong/2000/2009	Government Virus Unit	Centers for Disease Control and Prevention	-
	EPI397688	HA	Hong Kong (SAR)	2005-Jun-24	A/Hong Kong/4443/2005	Government Virus Unit	National Institute for Medical Research	-
	EPI335923	HA	United States	2010-Dec-30	A/Iowa/19/2010	Iowa State Hygienic Laboratory	Centers for Disease Control and Prevention	-
	EPI335922	NA	United States	2010-Dec-30	A/Iowa/19/2010	Iowa State Hygienic Laboratory	Centers for Disease Control and Prevention	-
	EPI211546	HA	South Africa	2008-Jun-26	A/Johannesburg/15/2008	National Institute for Communicable Disease	National Institute for Medical Research	-
	EPI211545	NA	South Africa	2008-Jun-26	A/Johannesburg/15/2008	National Institute for Communicable Disease	National Institute for Medical Research	-
	EPI405934	HA	South Africa	2012-Jun-04	A/Johannesburg/3224/2012	Sandringham, National Institute for Communicable D	National Institute for Medical Research	-
	EPI405935	NA	South Africa	2012-Jun-04	A/Johannesburg/3224/2012	Sandringham, National Institute for Communicable D	National Institute for Medical Research	-
	EPI210086	HA	South Africa	2009-Jun-08	A/Johannesburg/385/2009	National Institute for Communicable Disease	National Institute for Medical Research	-
	EPI210175	NA	South Africa	2009-Jun-08	A/Johannesburg/385/2009	National Institute for Communicable Disease	National Institute for Medical Research	-
	EPI232568	HA	Kenya	2009-Sep-15	A/Kenya/4408/2009	CDC-Kenya	Centers for Disease Control and Prevention	-
	EPI232567	NA	Kenya	2009-Sep-15	A/Kenya/4408/2009	CDC-Kenya	Centers for Disease Control and Prevention	-
	EPI606852	HA	Japan	2002-Dec-25	A/Kumamoto/102/2002	-	Other Database Import	Bedford,T.; Riley,S.; Barr,I.G.; Broor,S.; Chadha,M.; *et al.*
	EPI319276	HA	Madagascar	2011-Feb-21	A/Madagascar/0648/2011	Institut Pasteur de Madagascar	National Institute for Medical Research	-
	EPI319277	NA	Madagascar	2011-Feb-21	A/Madagascar/0648/2011	Institut Pasteur de Madagascar	National Institute for Medical Research	-
	EPI408608	HA	United States	2012-Nov-13	A/Maryland/43/2012	Maryland Department of Health and Mental Hygiene	Centers for Disease Control and Prevention	-
	EPI408607	NA	United States	2012-Nov-13	A/Maryland/43/2012	Maryland Department of Health and Mental Hygiene	Centers for Disease Control and Prevention	-
	EPI390181	HA	Nepal	2006-Aug-01	A/Nepal/921/2006	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI390183	NA	Nepal	2006-Aug-01	A/Nepal/921/2006	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI381971	HA	United States	2004-Jan-01	A/New York/55/2004	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI381973	NA	United States	2004-Jan-01	A/New York/55/2004	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI326137	HA	Norway	2011-Mar-16	A/Norway/1186/2011	Norwegian Institute of Public Health	National Institute for Medical Research	-
	EPI326138	NA	Norway	2011-Mar-16	A/Norway/1186/2011	Norwegian Institute of Public Health	National Institute for Medical Research	-
	EPI302231	HA	Norway	2010-Dec-03	A/Norway/1330/2010	WHO National Influenza Centre	National Institute for Medical Research	-
	EPI302230	NA	Norway	2010-Dec-03	A/Norway/1330/2010	WHO National Influenza Centre	National Institute for Medical Research	-

(Table S4.2 cont.)

Influenza (sub)type	Accession number	Segment	Country	Collection date (year-month-day)	Reference virus	Originating Lab	Submitting Lab	Authors
A(H3N2)	EPI384820	HA	United States	2012-Mar-08	A/Ohio/02/2012	WHO Collaborating Centre for Reference and Research on Influenza	Centers for Disease Control and Prevention	-
	EPI387810	NA	United States	2012-Mar-08	A/Ohio/02/2012	WHO Collaborating Centre for Reference and Research on Influenza	Centers for Disease Control and Prevention	-
	EPI417031	HA	France	2012-Oct-29	A/Paris/1651/2012	Institut Pasteur	National Institute for Medical Research	-
	EPI417032	NA	France	2012-Oct-29	A/Paris/1651/2012	Institut Pasteur	National Institute for Medical Research	-
	EPI215031	HA	France	2008-Dec-15	A/Paris/777/2008	Institut Pasteur	National Institute for Medical Research	-
	EPI215030	NA	France	2008-Dec-15	A/Paris/777/2008	Institut Pasteur	National Institute for Medical Research	-
	EPI390117	HA	Australia	2010-Aug-01	A/Perth/10/2010	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI390119	NA	Australia	2010-Aug-01	A/Perth/10/2010	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI182941	HA	Australia	2009-Apr-07	***A/Perth/16/2009***	Pathwest QE II Medical Centre	WHO Collaborating Centre for Reference and Research on Influenza	Deng,Y-M; Iannello,P; Erneste,J; Komadina,N.
	EPI182942	NA	Australia	2009-Apr-07	***A/Perth/16/2009***	Pathwest QE II Medical Centre	WHO Collaborating Centre for Reference and Research on Influenza	Deng,Y-M; Iannello,P; Erneste,J; Komadina,N.
	EPI390310	HA	United States	2010-Aug-01	A/Rhode Island/01/2010	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI390379	NA	United States	2010-Aug-01	A/Rhode Island/01/2010	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI326115	HA	Serbia	2011-Jan-06	A/Serbia/71/2011	Institute of Immunology and Virology Torlak	National Institute for Medical Research	-
	EPI326116	NA	Serbia	2011-Jan-06	A/Serbia/71/2011	Institute of Immunology and Virology Torlak	National Institute for Medical Research	-
	EPI155916	HA	Greece	2007-Jan-01	A/Serres/77/2007	-	Centers for Disease Control and Prevention	-
	EPI155915	NA	Greece	2007-Jan-01	A/Serres/77/2007	-	Centers for Disease Control and Prevention	-
	EPI545334	HA	Singapore	2004-Nov-01	A/Singapore/37/2004	-	Other Database Import	Fonville,J.M.; Wilks,S.H.; James,S.L.; Fox,A.; Ventresca,M.; *et al.*
	EPI117105	NA	Singapore	2004-Jun-07	A/Singapore/37/2004	-	Other Database Import	-
	EPI215043	HA	Singapore	2008-Sep-15	A/Singapore/N593/2008	Tan Tock Seng Hospital	National Institute for Medical Research	-
	EPI215042	NA	Singapore	2008-Sep-15	A/Singapore/N593/2008	Tan Tock Seng Hospital	National Institute for Medical Research	-
	EPI155918	HA	Bulgaria	2007-Jan-01	A/Sofia/319/2007	-	Centers for Disease Control and Prevention	-
	EPI155917	NA	Bulgaria	2007-Jan-01	A/Sofia/319/2007	-	Centers for Disease Control and Prevention	-
	EPI326139	HA	Sweden	2011-Mar-28	A/Stockholm/18/2011	Swedish Institute for Infectious Disease Control	National Institute for Medical Research	-
	EPI326140	NA	Sweden	2011-Mar-28	A/Stockholm/18/2011	Swedish Institute for Infectious Disease Control	National Institute for Medical Research	-
	EPI238690	HA	Sweden	2009-Aug-01	A/Stockholm/90/2009	Swedish Institute for Infectious Disease Control	Swedish Institute for Infectious Disease Control	-
	EPI238691	NA	Sweden	2009-Aug-01	A/Stockholm/90/2009	Swedish Institute for Infectious Disease Control	Swedish Institute for Infectious Disease Control	-
	EPI176951	HA	Australia	1997-Jan-01	***A/Sydney/05/1997***	-	Other Database Import	-
	EPI176953	NA	Australia	1997-Jan-01	***A/Sydney/05/1997***	-	Other Database Import	-
	EPI556816	HA	United States	2012-Apr-15	***A/Texas/50/2012***	Texas Department of State Health Services-Laboratory Services	Centers for Disease Control and Prevention	-
	EPI556815	NA	United States	2012-Apr-15	***A/Texas/50/2012***	Texas Department of State Health Services-Laboratory Services	Centers for Disease Control and Prevention	-
	EPI152544	HA	Uruguay	2007-Jan-01	A/Uruguay/716/2007	-	Centers for Disease Control and Prevention	-
	EPI152546	NA	Uruguay	2007-Jan-01	A/Uruguay/716/2007	-	Centers for Disease Control and Prevention	-
	EPI232629	HA	United States	2009-Sep-30	A/Vermont/22/2009	Vermont Department of Health Laboratory	Centers for Disease Control and Prevention	-
	EPI232628	NA	United States	2009-Sep-30	A/Vermont/22/2009	Vermont Department of Health Laboratory	Centers for Disease Control and Prevention	-
	EPI190148	HA	Australia	2009-Jun-02	A/Victoria/210/2009	Victorian Infectious Diseases Reference Laboratory	WHO Collaborating Centre for Reference and Research on Influenza	-
	EPI190149	NA	Australia	2009-Jun-02	A/Victoria/210/2009	Victorian Infectious Diseases Reference Laboratory	WHO Collaborating Centre for Reference and Research on Influenza	-
	EPI418017	HA	Australia	2011-Oct-24	***A/Victoria/361/2011***	WHO Collaborating Centre for Reference and Research on Influenza	Centers for Disease Control and Prevention	-
	EPI438985	NA	Australia	2011-Oct-24	***A/Victoria/361/2011***	WHO Collaborating Centre for Reference and Research on Influenza	Centers for Disease Control and Prevention	-
	EPI40980	HA	New Zealand	2005-Sep-13	A/Waikato/12/2005	-	Other Database Import	-
	EPI40985	NA	New Zealand	2005-Sep-13	A/Waikato/12/2005	-	Other Database Import	-
	EPI185937	HA	United States	2009-Apr-06	A/Washington/15/2009	Washington State Public Health Laboratory	Centers for Disease Control and Prevention	-
	EPI185935	NA	United States	2009-Apr-06	A/Washington/15/2009	Washington State Public Health Laboratory	Centers for Disease Control and Prevention	-
	EPI416181	HA	United States	2012-Dec-05	A/Washington/60/2012	Spokane Regional Health District	Centers for Disease Control and Prevention	-
	EPI416180	NA	United States	2012-Dec-05	A/Washington/60/2012	Spokane Regional Health District	Centers for Disease Control and Prevention	-
	EPI390109	HA	New Zealand	2004-Aug-01	A/Wellington/01/2004	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI390111	NA	New Zealand	2004-Aug-01	A/Wellington/01/2004	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI390189	HA	United States	2007-Aug-01	A/Wisconsin/03/2007	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI390191	NA	United States	2007-Aug-01	A/Wisconsin/03/2007	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI381901	HA	United States	2009-Aug-01	A/Wisconsin/15/2009	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI211309	NA	United States	2009-Jun-07	A/Wisconsin/15/2009	Wisconsin State Laboratory of Hygiene	Centers for Disease Control and Prevention	-
	EPI123261	HA	United States	2006-Dec-12	A/Wisconsin/42/2006	-	Centers for Disease Control and Prevention	-
	EPI123152	NA	United States	2006-Dec-12	A/Wisconsin/42/2006	-	Centers for Disease Control and Prevention	-
	EPI367110	HA	United States	2005-Jan-01	***A/Wisconsin/67/2005***	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI367112	NA	United States	2005-Jan-01	***A/Wisconsin/67/2005***	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI609838	HA	China	2004-Aug-23	A/Wuhan/269/2004	-	Other Database Import	Bedford,T.; Riley,S.; Barr,I.G.; Broor,S.; Chadha,M.; *et al.*
	EPI158311	NA	China	2004-Aug-23	A/Wuhan/269/2004	-	Centers for Disease Control and Prevention	-
	EPI397298	HA	China	1995-Oct-01	***A/Wuhan/359/1995***	-	Other Database Import	Hoschler,K.; Thomson,C.; Casas,I.; Ellis,J.; Galiano,M.; *et al.*
	EPI362822	NA	China	1995-Jan-01	***A/Wuhan/359/1995***	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI499365	HA	United States	2003-Feb-13	A/Wyoming/03/2003	Iowa State Hygienic Laboratory	Centers for Disease Control and Prevention	-
	EPI499364	NA	United States	2003-Feb-13	A/Wyoming/03/2003	Iowa State Hygienic Laboratory	Centers for Disease Control and Prevention	-
	EPI155914	HA	Croatia	2007-Jan-01	A/Zagreb/1216/2007	-	Centers for Disease Control and Prevention	-
	EPI155913	NA	Croatia	2007-Jan-01	A/Zagreb/1216/2007	-	Centers for Disease Control and Prevention	-

(Table S4.2 cont.)

Influenza (sub)type	Accession number	Segment	Country	Collection date (year-month-day)	Reference virus	Originating Lab	Submitting Lab	Authors
B	EPI279851	HA	Algeria	2010-Jun-06	B/Algeria/G486/2010	Institut Pasteur d'Algerie	National Institute for Medical Research	-
	EPI279850	NA	Algeria	2010-Jun-06	B/Algeria/G486/2010	Institut Pasteur d'Algerie	National Institute for Medical Research	-
	EPI352447	HA	Cote d'Ivoire	2011-Sep-22	B/Attecoube/GR887/2011	Pasteur Institut of Côte d'Ivoire	National Institute for Medical Research	-
	EPI352448	NA	Cote d'Ivoire	2011-Sep-22	B/Attecoube/GR887/2011	Pasteur Institut of Côte d'Ivoire	National Institute for Medical Research	-
	EPI416443	HA	Germany	2012-Nov-26	B/Baden Wurttemberg/41/2012	Robert-Koch-Institute	National Institute for Medical Research	-
	EPI416444	NA	Germany	2012-Nov-26	B/Baden Wurttemberg/41/2012	Robert-Koch-Institute	National Institute for Medical Research	-
	EPI366548	HA	Bangladesh	2007-Jan-01	B/Bangladesh/3333/2007	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI366549	NA	Bangladesh	2007-Jan-01	B/Bangladesh/3333/2007	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI352417	HA	Germany	2011-Oct-19	B/Berlin/146/2011	Robert-Koch-Institute	National Institute for Medical Research	-
	EPI352418	NA	Germany	2011-Oct-19	B/Berlin/146/2011	Robert-Koch-Institute	National Institute for Medical Research	-
	EPI294717	HA	Bolivia, Plurinationial State of	2010-Jan-01	B/Bolivia/1526/2010	-	Centers for Disease Control and Prevention	-
	EPI294716	NA	Bolivia, Plurinationial State of	2010-Jan-01	B/Bolivia/1526/2010	-	Centers for Disease Control and Prevention	-
	EPI168165	HA	Brazil	2008-Jul-28	B/Brazil/2937/2008	-	Centers for Disease Control and Prevention	-
	EPI168274	NA	Brazil	2008-Jul-28	B/Brazil/2937/2008	-	Centers for Disease Control and Prevention	-
	EPI155964	HA	Australia	2007-Mar-09	B/Brisbane/03/2007	-	Centers for Disease Control and Prevention	-
	EPI155963	NA	Australia	2007-Mar-09	B/Brisbane/03/2007	-	Centers for Disease Control and Prevention	-
	EPI163726	HA	Australia	2008-Jul-13	B/Brisbane/33/2008	Queensland Health Scientific Services	WHO Collaborating Centre for Reference and Research on Influenza	-
	EPI186302	NA	Australia	2008-Jul-13	B/Brisbane/33/2008	Queensland Health Scientific Services	WHO Collaborating Centre for Reference and Research on Influenza	-
	EPI163725	HA	Australia	2008-Aug-04	***B/Brisbane/60/2008***	Queensland Health Scientific Services	WHO Collaborating Centre for Reference and Research on Influenza	-
	EPI173276	NA	Australia	2008-Aug-04	***B/Brisbane/60/2008***	Queensland Health Scientific Services	WHO Collaborating Centre for Reference and Research on Influenza	-
	EPI348438	HA	Cambodia	2011-Oct-10	B/Cambodia/1412/2011	National Public Health Laboratory	Centers for Disease Control and Prevention	-
	EPI348437	NA	Cambodia	2011-Oct-10	B/Cambodia/1412/2011	National Public Health Laboratory	Centers for Disease Control and Prevention	-
	EPI366564	HA	United Kingdom	2008-Jan-01	B/England/145/2008	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI366566	NA	United Kingdom	2008-Jan-01	B/England/145/2008	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI211559	HA	United Kingdom	2008-Aug-29	B/England/393/2008	Health Protection Agency	National Institute for Medical Research	-
	EPI211560	NA	United Kingdom	2008-Aug-29	B/England/393/2008	Health Protection Agency	National Institute for Medical Research	-
	EPI315675	HA	United Kingdom	2007-Oct-03	B/England/552/2007	-	Other Database Import	Ellis,J.
	EPI279859	HA	United Kingdom	2010-May-02	B/England/63/2010	Microbiology Services Colindale, Public Health England	National Institute for Medical Research	-
	EPI279858	NA	United Kingdom	2010-May-02	B/England/63/2010	Microbiology Services Colindale, Public Health England	National Institute for Medical Research	-
	EPI406983	HA	Estonia	2011-Mar-14	B/Estonia/55669/2011	National Institute for Medical Research	Centers for Disease Control and Prevention	-
	EPI406982	NA	Estonia	2011-Mar-14	B/Estonia/55669/2011	National Institute for Medical Research	Centers for Disease Control and Prevention	-
	EPI294743	HA	United States	2010-Oct-18	B/Florida/04/2010	Florida Department of Health-Jacksonville	Centers for Disease Control and Prevention	-
	EPI294742	NA	United States	2010-Oct-18	B/Florida/04/2010	Florida Department of Health-Jacksonville	Centers for Disease Control and Prevention	-
	EPI159978	HA	United States	2006-Jan-01	***B/Florida/04/2006***	-	Other Database Import	-
	EPI159980	NA	United States	2006-Jan-01	***B/Florida/04/2006***	-	Other Database Import	-
	EPI406250	HA	Argentina	2012-Aug-06	B/Formosa/V2367/2012	Instituto Nacional de Enfermedades Infecciosas	National Institute for Medical Research	-
	EPI406251	NA	Argentina	2012-Aug-06	B/Formosa/V2367/2012	Instituto Nacional de Enfermedades Infecciosas	National Institute for Medical Research	-
	EPI366580	HA	China	2008-Jan-01	B/Fujian-Gulou/1272/2008	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI366582	NA	China	2008-Jan-01	B/Fujian-Gulou/1272/2008	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI51231	HA	United States	2004-Jun-21	B/Hawaii/13/2004	-	Other Database Import	-
	EPI51236	NA	United States	2004-Jun-21	B/Hawaii/13/2004	-	Other Database Import	-
	EPI156987	HA	Honduras	2005-Dec-20	B/Honduras/1465/2005	-	Centers for Disease Control and Prevention	-
	EPI301202	HA	Honduras	2010-Oct-11	B/Honduras/6927/2010	Laboratorio Nacional de Virologia	Centers for Disease Control and Prevention	-
	EPI301201	NA	Honduras	2010-Oct-11	B/Honduras/6927/2010	Laboratorio Nacional de Virologia	Centers for Disease Control and Prevention	-
	EPI105163	HA	Hong Kong (SAR)	1972-Jan-01	***B/Hong Kong/05/1972***	-	Other Database Import	-
	EPI105167	NA	Hong Kong (SAR)	1972-Jan-01	***B/Hong Kong/05/1972***	-	Other Database Import	-
	EPI392575	HA	Hong Kong (SAR)	2012-Apr-30	B/Hong Kong/3496/2012	Government Virus Unit	National Institute for Medical Research	-
	EPI392576	NA	Hong Kong (SAR)	2012-Apr-30	B/Hong Kong/3496/2012	Government Virus Unit	National Institute for Medical Research	-
	EPI392589	HA	Hong Kong (SAR)	2012-Jun-13	B/Hong Kong/3577/2012	Government Virus Unit	National Institute for Medical Research	-
	EPI392590	NA	Hong Kong (SAR)	2012-Jun-13	B/Hong Kong/3577/2012	Government Virus Unit	National Institute for Medical Research	-
	EPI211555	HA	Hong Kong (SAR)	2005-Feb-07	B/Hong Kong/45/2005	Government Virus Unit	National Institute for Medical Research	-
	EPI211556	NA	Hong Kong (SAR)	2005-Feb-07	B/Hong Kong/45/2005	Government Virus Unit	National Institute for Medical Research	-
	EPI377713	HA	Ireland	2012-Feb-29	B/Ireland/12M17522/2012	National Virus Reference Laboratory	National Institute for Medical Research	-
	EPI377714	NA	Ireland	2012-Feb-29	B/Ireland/12M17522/2012	National Virus Reference Laboratory	National Institute for Medical Research	-
	EPI352840	HA	Israel	2011-Dec-13	B/Israel/15/2011	Central Virology Laboratory Israel (NIC)	National Institute for Medical Research	-
	EPI352841	NA	Israel	2011-Dec-13	B/Israel/15/2011	Central Virology Laboratory Israel (NIC)	National Institute for Medical Research	-
	EPI187537	HA	China	2009-Feb-23	B/Jiangsu-Gulou/125/2009	WHO Chinese National Influenza Center	Centers for Disease Control and Prevention	-
	EPI187538	NA	China	2009-Feb-23	B/Jiangsu-Gulou/125/2009	WHO Chinese National Influenza Center	Centers for Disease Control and Prevention	-
	CY033844*	HA	China	2003	B/Jiangsu/10/2003	-	-	Spiro,D., Halpin,R., Boyne,A., Bera,J., Ghedin,E., *et al.*
	CY033846*	NA	China	2003	B/Jiangsu/10/2003	-	-	-
	EPI159930	HA	China	2003-Jan-01	B/Jilin/20/2003	-	Other Database Import	-
	EPI159932	NA	China	2003-Jan-01	B/Jilin/20/2003	-	Other Database Import	-
	EPI406272	HA	South Africa	2012-Aug-03	B/Johannesburg/3964/2012	Sandringham, National Institute for Communicable D	National Institute for Medical Research	-
	EPI406273	NA	South Africa	2012-Aug-03	B/Johannesburg/3964/2012	Sandringham, National Institute for Communicable D	National Institute for Medical Research	-
	EPI294801	HA	Lao, People's Democratic Republic	2010-Sep-14	B/Laos/833/2010	National Center for Laboratory and Epidemiology	Centers for Disease Control and Prevention	-
	EPI294800	NA	Lao, People's Democratic Republic	2010-Sep-14	B/Laos/833/2010	National Center for Laboratory and Epidemiology	Centers for Disease Control and Prevention	-

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Influenza (sub)type	Accession number	Segment	Country	Collection date (year-month-day)	Reference virus	Originating Lab	Submitting Lab	Authors
	EPI334664	HA	France	2011-Mar-01	B/Lyon/1179/2011	CRR virus Influenza region Sud	National Institute for Medical Research	-
	EPI334665	NA	France	2011-Mar-01	B/Lyon/1179/2011	CRR virus Influenza region Sud	National Institute for Medical Research	-
	EPI175755	HA	Malaysia	2004-Jan-01	***B/Malaysia/2506/2004***	-	Other Database Import	-
	EPI175757	NA	Malaysia	2004-Jan-01	***B/Malaysia/2506/2004***	-	Other Database Import	-
	EPI346734	HA	Malta	2011-Mar-07	B/Malta/MV636714/2011	Mater Dei Hospital	National Institute for Medical Research	-
	EPI346735	NA	Malta	2011-Mar-07	B/Malta/MV636714/2011	Mater Dei Hospital	National Institute for Medical Research	-
	EPI376344	HA	United States	2012-Mar-13	***B/Massachusetts/02/2012***	Massachusetts Department of Public Health	Centers for Disease Control and Prevention	-
	EPI376343	NA	United States	2012-Mar-13	***B/Massachusetts/02/2012***	Massachusetts Department of Public Health	Centers for Disease Control and Prevention	-
	EPI406961	HA	United States	2012-Oct-28	B/Montana/06/2012	Montana Laboratory Services Bureau	Centers for Disease Control and Prevention	-
	EPI406960	NA	United States	2012-Oct-28	B/Montana/06/2012	Montana Laboratory Services Bureau	Centers for Disease Control and Prevention	-
	EPI407195	HA	United States	2012-Nov-26	B/New Mexico/04/2012	New Mexico Department of Health	Centers for Disease Control and Prevention	-
	EPI407194	NA	United States	2012-Nov-26	B/New Mexico/04/2012	New Mexico Department of Health	Centers for Disease Control and Prevention	-
	EPI368752	HA	Russian Federation	2012-Feb-14	B/Novosibirsk/01/2012	Russian Academy of Medical Sciences	Centers for Disease Control and Prevention	-
	EPI368751	NA	Russian Federation	2012-Feb-14	B/Novosibirsk/01/2012	Russian Academy of Medical Sciences	Centers for Disease Control and Prevention	-
	EPI346478	HA	United States	2005-Feb-23	B/Ohio/01/2005	Cincinnati Children's Hospital	Centers for Disease Control and Prevention	-
	EPI346479	NA	United States	2005-Feb-23	B/Ohio/01/2005	Cincinnati Children's Hospital	Centers for Disease Control and Prevention	-
	AJ784057*	HA	Norway	2004	B/Oslo/71/2004	-	-	-
	AJ784101*	NA	Norway	2004	B/Oslo/71/2004	-	-	-
	EPI211562	HA	France	2009-Feb-09	B/Paris/1762/2009	Institut Pasteur	National Institute for Medical Research	-
	EPI211561	NA	France	2009-Feb-09	B/Paris/1762/2009	Institut Pasteur	National Institute for Medical Research	-
	EPI326171	HA	Serbia	2011-Mar-08	B/Serbia/1894/2011	Institute of Immunology and Virology Torlak	National Institute for Medical Research	-
	EPI326172	NA	Serbia	2011-Mar-08	B/Serbia/1894/2011	Institute of Immunology and Virology Torlak	National Institute for Medical Research	-
	EPI347893	HA	China	2011-Nov-12	B/Shanghai-Chongming/1458/2011	WHO Chinese National Influenza Center	WHO Chinese National Influenza Center	Lan, Y.; Li, X.;Zhao, X.; Cheng,Y.; Tan,M.; *et al.*
	EPI347892	NA	China	2011-Nov-12	B/Shanghai-Chongming/1458/2011	WHO Chinese National Influenza Center	WHO Chinese National Influenza Center	Lan, Y.; Li, X.;Zhao, X.; Cheng,Y.; Tan,M.; *et al.*
B	EPI13603	HA	China	2002-Jun-12	***B/Shanghai/361/2002***	-	Other Database Import	-
	EPI116668	NA	China	2002-Jun-12	***B/Shanghai/361/2002***	-	Other Database Import	-
	EPI131322	HA	Singapore	1979-Jan-01	***B/Singapore/222/1979***	-	Other Database Import	-
	EPI129892	NA	Singapore	1979-Jan-01	***B/Singapore/222/1979***	-	Other Database Import	-
	EPI301320	HA	Slovakia	2010-Jul-27	B/Slovakia/1731/2010	National Public Health Institute of Slovakia	National Institute for Medical Research	-
	EPI301319	NA	Slovakia	2010-Jul-27	B/Slovakia/1731/2010	National Public Health Institute of Slovakia	National Institute for Medical Research	-
	EPI340834	HA	Sweden	2011-Feb-28	B/Stockholm/12/2011	National Institute for Medical Research	Centers for Disease Control and Prevention	-
	EPI340833	NA	Sweden	2011-Feb-28	B/Stockholm/12/2011	National Institute for Medical Research	Centers for Disease Control and Prevention	-
	EPI438791	HA	Germany	2013-Jan-07	B/Thuringen/01/2013	Robert-Koch-Institute	National Institute for Medical Research	-
	EPI438792	NA	Germany	2013-Jan-07	B/Thuringen/01/2013	Robert-Koch-Institute	National Institute for Medical Research	-
	EPI233234	HA	Uganda	2009-Oct-21	B/Uganda/0514/2009	Uganda Virus Research Institute (UVRI), National Influenza Center	Centers for Disease Control and Prevention	-
	EPI233233	NA	Uganda	2009-Oct-21	B/Uganda/0514/2009	Uganda Virus Research Institute (UVRI), National Influenza Center	Centers for Disease Control and Prevention	-
	EPI211563	HA	Spain	2008-Feb-18	B/Valladolid/18/2008	Universidad de Valladolid	National Institute for Medical Research	-
	EPI211564	NA	Spain	2008-Feb-18	B/Valladolid/18/2008	Universidad de Valladolid	National Institute for Medical Research	-
	EPI51801	HA	Canada	1987-Jan-01	B/Victoria/02/1987	-	Other Database Import	-
	EPI51806	NA	Canada	1987-Jan-01	B/Victoria/02/1987	-	Other Database Import	-
	EPI502811	HA	Australia	2006-Jun-06	B/Victoria/304/2006	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI502813	NA	Australia	2006-Jun-06	B/Victoria/304/2006	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI483004	HA	Australia	2005-Oct-16	B/Victoria/530/2005	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI483006	NA	Australia	2005-Oct-16	B/Victoria/530/2005	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI271600	HA	United States	2010-Feb-20	***B/Wisconsin/01/2010***	Wisconsin State Laboratory of Hygiene	Centers for Disease Control and Prevention	-
	EPI271599	NA	United States	2010-Feb-20	***B/Wisconsin/01/2010***	Wisconsin State Laboratory of Hygiene	Centers for Disease Control and Prevention	-
	EPI43948	HA	United States	2006-Apr-02	B/Wisconsin/10/2006	-	Other Database Import	-
	EPI51820	HA	Japan	1988-Jan-01	***B/Yamagata/16/1988***	-	Other Database Import	-
	EPI51825	NA	Japan	1988-Jan-01	***B/Yamagata/16/1988***	-	Other Database Import	-
	EPI319590	HA	Russian Federation	2011-Feb-28	A/Astrakhan/01/2011	WHO National Influenza Centre Russian Federation	National Institute for Medical Research	-
	EPI319591	NA	Russian Federation	2011-Feb-28	A/Astrakhan/01/2011	WHO National Influenza Centre Russian Federation	National Institute for Medical Research	-
	EPI177661	HA	New Zealand	2009-Apr-25	A/Auckland/03/2009	Auckland Hospital	WHO Collaborating Centre for Reference and Research on Influenza	-
	EPI177280	NA	New Zealand	2009-Apr-25	A/Auckland/03/2009	Auckland Hospital	WHO Collaborating Centre for Reference and Research on Influenza	-
	EPI239631	HA	Germany	2009-Jan-01	A/Bayern/69/2009	-	Centers for Disease Control and Prevention	-
	EPI239630	NA	Germany	2009-Jan-01	A/Bayern/69/2009	-	Centers for Disease Control and Prevention	-
A(H1N1)pdm09	EPI177294	HA	United States	2009-Apr-09	***A/California/07/2009***	Naval Health Research Center	Centers for Disease Control and Prevention	-
	EPI185379	NA	United States	2009-Apr-09	***A/California/07/2009***	Naval Health Research Center	Centers for Disease Control and Prevention	-
	EPI280344	HA	New Zealand	2010-Jul-12	A/Christchurch/16/2010	WHO Collaborating Centre for Reference and Research on Influenza	Centers for Disease Control and Prevention	-
	EPI280343	NA	New Zealand	2010-Jul-12	A/Christchurch/16/2010	WHO Collaborating Centre for Reference and Research on Influenza	Centers for Disease Control and Prevention	-
	EPI319447	HA	Czech Republic	2011-Jan-18	A/Czech Republic/32/2011	National Institute of Public Health	National Institute for Medical Research	-
	EPI319448	NA	Czech Republic	2011-Jan-18	A/Czech Republic/32/2011	National Institute of Public Health	National Institute for Medical Research	-
	EPI417122	HA	Senegal	2012-Dec-09	A/Dakar/20/2012	Institut Pasteur de Dakar	National Institute for Medical Research	-
	EPI417123	NA	Senegal	2012-Dec-09	A/Dakar/20/2012	Institut Pasteur de Dakar	National Institute for Medical Research	-
	EPI190214	HA	Denmark	2009-Jun-09	A/Denmark/528/2009	-	Other Database Import	-
	EPI190216	NA	Denmark	2009-Jun-09	A/Denmark/528/2009	-	Other Database Import	-
	EPI294674	HA	United Kingdom	2010-Nov-01	A/England/4880374/2010	Microbiology Services Colindale, Public Health England	Microbiology Services Colindale, Public Health England	Galiano,M.
	EPI294673	NA	United Kingdom	2010-Nov-01	A/England/4880374/2010	Microbiology Services Colindale, Public Health England	Microbiology Services Colindale, Public Health England	Galiano,M.

(Table S4.2 cont.)

Influenza (sub)type	Accession number	Segment	Country	Collection date (year-month-day)	Reference virus	Originating Lab	Submitting Lab	Authors
A(H1N1)pdm09	EPI348185	HA	United States	2011-Dec-07	A/Florida/35/2011	Florida Department of Health-Tampa	Centers for Disease Control and Prevention	-
	EPI348184	NA	United States	2011-Dec-07	A/Florida/35/2011	Florida Department of Health-Tampa	Centers for Disease Control and Prevention	-
	EPI331061	HA	Ghana	2011-May-13	A/Ghana/763/2011	University of Ghana	National Institute for Medical Research	-
	EPI331062	NA	Ghana	2011-May-13	A/Ghana/763/2011	University of Ghana	National Institute for Medical Research	-
	EPI279895	HA	Hong Kong (SAR)	2010-Jul-16	A/Hong Kong/2212/2010	Government Virus Unit	National Institute for Medical Research	-
	EPI279894	NA	Hong Kong (SAR)	2010-Jul-16	A/Hong Kong/2212/2010	Government Virus Unit	National Institute for Medical Research	-
	EPI326206	HA	Hong Kong (SAR)	2011-Mar-29	A/Hong Kong/3934/2011	Government Virus Unit	National Institute for Medical Research	-
	EPI326207	NA	Hong Kong (SAR)	2011-Mar-29	A/Hong Kong/3934/2011	Government Virus Unit	National Institute for Medical Research	-
	EPI382424	HA	Hong Kong (SAR)	2012-May-21	A/Hong Kong/5659/2012	Public Health Laboratory Services Branch, Centre for Health Protection	Public Health Laboratory Services Branch, Centre for Health Protection	Mak,G.C.;Lo,J.Y.C.
	EPI382425	NA	Hong Kong (SAR)	2012-May-21	A/Hong Kong/5659/2012	Public Health Laboratory Services Branch, Centre for Health Protection	Public Health Laboratory Services Branch, Centre for Health Protection	Mak,G.C.;Lo,J.Y.C.
	EPI178946	HA	United States	2006-Nov-08	A/Iowa/01/2006	Iowa State Hygienic Laboratory	Centers for Disease Control and Prevention	-
	EPI342434	HA	United States	2010-Nov-01	A/Kentucky/09/2010	Kentucky Division of Laboratory Services	Centers for Disease Control and Prevention	-
	EPI342433	NA	United States	2010-Nov-01	A/Kentucky/09/2010	Kentucky Division of Laboratory Services	Centers for Disease Control and Prevention	-
	EPI346699	HA	France	2011-Feb-23	A/Lorraine/1176/2011	CRR virus Influenza region Sud	National Institute for Medical Research	-
	EPI346700	NA	France	2011-Feb-23	A/Lorraine/1176/2011	CRR virus Influenza region Sud	National Institute for Medical Research	-
	EPI215957	HA	Ukraine	2009-Oct-27	A/Lviv/N6/2009	Ministry of Health of Ukraine	National Institute for Medical Research	-
	EPI215956	NA	Ukraine	2009-Oct-27	A/Lviv/N6/2009	Ministry of Health of Ukraine	National Institute for Medical Research	-
	EPI416401	HA	France	2012-Dec-28	A/Lyon/02/2013	CRR virus Influenza region Sud	National Institute for Medical Research	-
	EPI416402	NA	France	2012-Dec-28	A/Lyon/02/2013	CRR virus Influenza region Sud	National Institute for Medical Research	-
	EPI408003	HA	United States	1976-Jan-01	A/New Jersey/8/1976	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Wester,E.; *et al.*
	EPI233202	HA	United States	2009-Oct-16	A/North Carolina/49/2009	North Carolina State Laboratory of Public Health	Centers for Disease Control and Prevention	-
	EPI233201	NA	United States	2009-Oct-16	A/North Carolina/49/2009	North Carolina State Laboratory of Public Health	Centers for Disease Control and Prevention	-
	EPI416419	HA	Norway	2012-Dec-17	A/Norway/2552/2012	WHO National Influenza Centre	National Institute for Medical Research	-
	EPI416420	NA	Norway	2012-Dec-17	A/Norway/2552/2012	WHO National Influenza Centre	National Institute for Medical Research	-
	EPI189161	HA	Singapore	2009-May-30	A/Singapore/57/2009	Singapore General Hospital	Centers for Disease Control and Prevention	-
	EPI189162	NA	Singapore	2009-May-30	A/Singapore/57/2009	Singapore General Hospital	Centers for Disease Control and Prevention	-
	EPI346513	HA	Thailand	2011-Sep-12	A/Song Khla/270/2011	WHO National Influenza Centre, National Institute of Medical Research (NIMR)	Centers for Disease Control and Prevention	-
	EPI346512	NA	Thailand	2011-Sep-12	A/Song Khla/270/2011	WHO National Influenza Centre, National Institute of Medical Research (NIMR)	Centers for Disease Control and Prevention	-
	EPI316435	HA	Russian Federation	2011-Mar-14	A/St. Petersburg/100/2011	Russian Academy of Medical Sciences	Centers for Disease Control and Prevention	-
	EPI316434	NA	Russian Federation	2011-Mar-14	A/St. Petersburg/100/2011	Russian Academy of Medical Sciences	Centers for Disease Control and Prevention	-
	EPI319527	HA	Russian Federation	2011-Feb-14	A/St. Petersburg/27/2011	WHO National Influenza Centre Russian Federation	National Institute for Medical Research	-
	EPI319528	NA	Russian Federation	2011-Feb-14	A/St. Petersburg/27/2011	WHO National Influenza Centre Russian Federation	National Institute for Medical Research	-
	EPI301380	HA	Sweden	2010-Dec-02	A/Stockholm/12/2010	Swedish Institute for Infectious Disease Control	Swedish Institute for Infectious Disease Control	-
	EPI301381	NA	Sweden	2010-Dec-02	A/Stockholm/12/2010	Swedish Institute for Infectious Disease Control	Swedish Institute for Infectious Disease Control	-
	EPI301779	HA	Sweden	2010-Dec-12	A/Stockholm/14/2010	Swedish Institute for Infectious Disease Control	Swedish Institute for Infectious Disease Control	-
	EPI301780	NA	Sweden	2010-Dec-12	A/Stockholm/14/2010	Swedish Institute for Infectious Disease Control	Swedish Institute for Infectious Disease Control	-
	EPI347564	HA	Sweden	2011-Nov-22	A/Stockholm/35/2011	-	Swedish Institute for Infectious Disease Control	-
	EPI347565	NA	Sweden	2011-Nov-22	A/Stockholm/35/2011	-	Swedish Institute for Infectious Disease Control	-
	EPI396874	NA	France	1982-Oct-01	A/swine/Finnistere/2899/1982	-	Other Database Import	Lycett,S.J.; Baillie,G.; Coulter,E.; Bhatt,S.; Kellam,P.; *et al.*
	EPI396914	NA	France	1999-Oct-01	A/swine/Ille et Vilaine/1455/1999	-	Other Database Import	Lycett,S.J.; Baillie,G.; Coulter,E.; Bhatt,S.; Kellam,P.; *et al.*
	EPI12290	NA	Italy	1995-Jan-01	A/swine/Italy/1424-4/1995	-	Other Database Import	-
	EPI396960	NA	Italy	1998-Oct-01	A/swine/Italy/1513-1/1998	-	Other Database Import	Lycett,S.J.; Baillie,G.; Coulter,E.; Bhatt,S.; Kellam,P.; *et al.*
	EPI173655	HA	United States	2007-Jan-01	A/swine/OH/511445/2007	-	Other Database Import	-
	EPI406026	HA	United States	2012-Oct-31	A/Tennessee/09/2012	Tennessee Department of Health Laboratory-Nashville	Centers for Disease Control and Prevention	-
	EPI406025	NA	United States	2012-Oct-31	A/Tennessee/09/2012	Tennessee Department of Health Laboratory-Nashville	Centers for Disease Control and Prevention	-
	EPI335840	HA	Chile	2011-Aug-10	A/Valparaiso/17275/2011	Instituto de Salud Publica de Chile	Centers for Disease Control and Prevention	-
	EPI335839	NA	Chile	2011-Aug-10	A/Valparaiso/17275/2011	Instituto de Salud Publica de Chile	Centers for Disease Control and Prevention	-
	EPI239643	HA	Vietnam	2009-Dec-01	A/Vietnam/2043/2009	National Institute of Hygiene and Epidemiology	Centers for Disease Control and Prevention	-
	EPI239642	NA	Vietnam	2009-Dec-01	A/Vietnam/2043/2009	National Institute of Hygiene and Epidemiology	Centers for Disease Control and Prevention	-
	EPI247506	HA	United States	2010-Feb-08	A/Wyoming/01/2010	Wyoming Public Health Laboratory	Centers for Disease Control and Prevention	-
	EPI247505	NA	United States	2010-Feb-08	A/Wyoming/01/2010	Wyoming Public Health Laboratory	Centers for Disease Control and Prevention	-

NA: Neuraminidase; HA: Hemagglutinin

Virtually all sequences were retrieved from GISAID EpiFlu™ database. The four influenza B virus sequences (2 HA+2NA) retrieved from NCBI Influenza Virus Resource database are marked with an asterisk (*) after the accession number (2nd column). Reference viruses included in the Northern Hemisphere´s seasonal influenza vaccines are highlighted in bold and italic. The dash (-) represents no information.

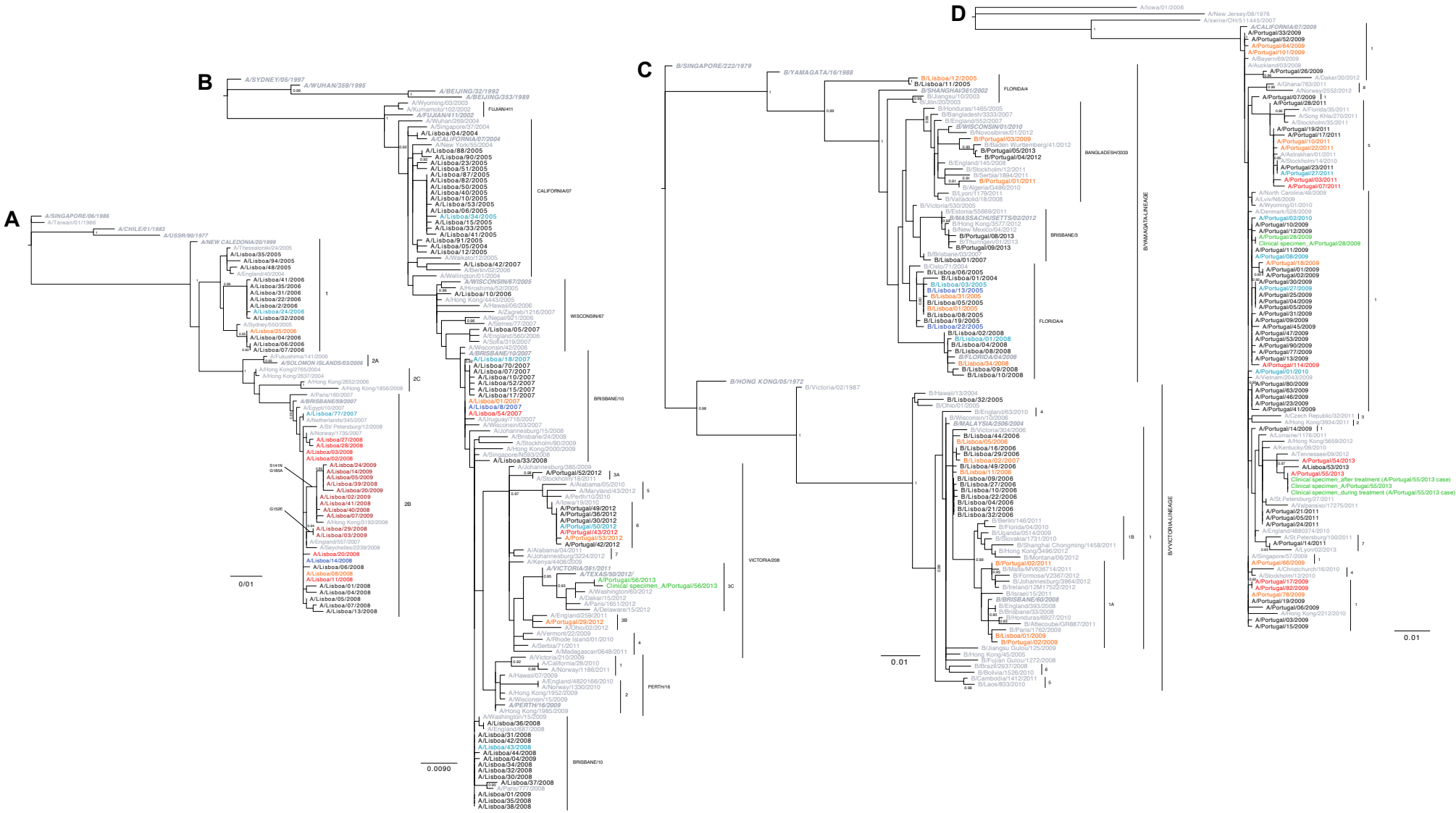

Figure S4.1 Phylogenetic comparison of former seasonal A(H1N1) **(A)**, A(H3N2) **(B)**, influenza B **(C)** and A(H1N1)pdm09 virus **(D)** hemagglutinin genes.

Hemagglutinin (HA) phylogenetic trees were constructed and annotated as described in Figure 4.11 A, with influenza A(H3N2) (panel B) and A(H1N1)pdm09 (panel D) upper extreme outlier viruses being just coloured in light red. Influenza A(H3N2) (panel B), B (panel C) and A(H1N1)pdm09 (panel D) HA genetic clades are described in Figure 4.13.

CHAPTER 5

WHOLE-GENOME MUTATIONAL LANDSCAPE OF INFLUENZA VIRUSES RESISTANT OR WITH DECREASED SUSCEPTIBILITY TO OSELTAMIVIR AND/OR ZANAMIVIR

Some results published in:

Gíria M, Rebelo-de-Andrade H, Santos LA, **Correia V**, Pedro S, Santos MM, 2012. Genomic signatures and antiviral drug susceptibility profile of A(H1N1)pdm09. J Clin Virol 53(2), 140-144.

Santos LA, **Correia V**, Giria M, Pedro S, Santos MM, Silvestre MJ, Rebelo-de-Andrade H, 2011. Genetic and Antiviral Drug Susceptibility Profiles of Pandemic A(H1N1)v Influenza Virus Circulating in Portugal. Influenza Other Respi Viruses 5 (Suppl. 1), 294–300.

I carried out all activities, methodologies and data analysis underlying the results presented in this chapter. Gene sequences of A(H1N1)pdm09 viruses sensitive to oseltamivir and zanamivir were included as were available at the laboratory sequence database, having been obtained by two team colleagues to study the molecular dynamics underlying the evolution of the newly emergent A(H1N1) pandemic virus.

FCG-funding research project

"*Avaliação e Caracterização da Emergência das Resistências aos Antivirais Específicos para a Gripe no Contexto da Infecção Respiratória Aguda*" (SDH49; ACSS reinforcement grant)

5 WHOLE-GENOME MUTATIONAL LANDSCAPE OF INLUENZA VIRUSES RESISTANT OR WITH DECREASED SUSCEPTIBILITY TO OSELTAMIVIR AND/OR ZANAMIVIR

"Evolution will outsmart intelligent design every time."

Steitz T. Lunchtime science. Nature 2011; 478:S2–S3.

This is a short-completing chapter of the previous one (Chapter 4). It presents and discusses the results of the genome-wide mutational analysis performed on the influenza viruses identified as resistant or with decreased susceptibility to oseltamivir (OS) and/or zanamivir (ZA) (2nd general objective; see 2.2, Study Description). Neuraminidase (NA) and hemagglutinin (HA) virus genome segments (segments 6 and 4, respectively) are not presented as were already covered in the previous chapter.

The main activities carried out during this study are summarized below.

BOX 5.1 - MAIN ACTIVITIES

- Implementation and optimization of a Sanger sequencing protocol for former seasonal A(H1N1), A(H3N2) and influenza B virus whole-genome.
- Whole-genome sequencing of influenza viruses exhibiting resistance or decreased susceptibility to OS and/or ZA - PB2, PB1, PA, NP, M and NS segments.
- Mutational analysis against the influenza virus type- or subtype-matched consensus sequence.

5.1 RESULTS

Thirty-eight human influenza viruses exhibiting resistance or decreased susceptibility to OS and/or ZA (14 former seasonal A(H1N1), 3 A(H3N2), 8 B, and 13 A(H1N1)pdm09) were evaluated through whole-genome sequencing (PB2, PB1, PA, NP, M and NS segments). This included most (38/63) influenza viruses identified as resistant or with decreased susceptibility in previous antiviral susceptibility testing surveillance activities (see Chapter 4). Due to the overall OS resistance observed for former seasonal A(H1N1) subtype (herein designated as seasonal A(H1N1)) in 2008/2009, only ~25% (6/21) of the NA H275Y OS-resistant viruses from that season, distributed over time, were

selected for whole-genome characterization. Also, for 10 of the viruses of the other influenza types or subtypes exhibiting decreased susceptibility to the drug(s), it was not available enough volume of viral isolate to perform the characterization (2 A(H3N2), 5 B, and 3 A(H1N1)pdm09).

Table 5.1 presents the amino acid substitutions found specifically in the PB2 (PB2 protein), PB1 (PB1, PB1-N40, and PB1-F2 proteins), PA (PA, PA-N155, PA-N182, and PA-X proteins), NP (NP protein), M (M1, and M2 or BM2 proteins), and NS (NS1 and NEP proteins) genome segments of the influenza viruses resistant or with decreased susceptibility to OS and/or ZA, compared to the virus type or subtype-matched consensus sequence.

NA H275Y OS-resistant seasonal A(H1N1) viruses from 2007/2008 and 2008/2009 shared two amino acid substitutions located in two different genome segments - PB2 P453S and PB1-F2 H3P. Both substitutions were exceptionally identified against the sequence of the clade 2B OS-sensitive reference virus A/Brisbane/59/2007. The NA H275Y viruses from 2008/2009 shared also the N642S amino acid substitution in PB1 protein (N603S in PB1-N40 protein). Structurally, residue 453 belongs to the PB2 cap-binding domain[A] but is located distantly from the residues involved in the binding to the 5´cap of host messenger RNAs (mRNAs) (cap-binding residues), as evidenced in Figure 5.1A. Residue 642 is located outside of all functional domains described to date for PB1[B], and the PB1-F2 protein of former circulating A(H1N1) viruses is present in the C-terminally truncated form of 19 amino acids that is thought to be non-functional (known functions of PB1-F2 are mostly associated with the C-terminal end of the protein) [3]. The NA H275Y OS-resistant seasonal A(H1N1) virus A/Lisboa/24/2009 contained two amino acid substitutions in or close to residues functionally important. Specifically, the F254V substitution in the PB1 3´vRNA binding RNP1 motif (residues 251-254)[B], and the PA I596V substitution adjacently to residue M595 involved in PB1 binding (PA PB1-binding domain[C]; Figure 5.1F). It also contained the Y89P amino acid substitution in the region of the non-structural protein 1 (NS1) that interacts with the host-cell factor eIF4GI involved in viral mRNA translation (residues 81-113) [4]. The PA I596V amino acid substitution was

[A] Domain structure of influenza A virus PB2 protein based on Mehle and McCullers [1].
[B] Domain structure and critical residues of influenza A virus PB1 protein based on Mehle and McCullers [1] and Chu *et al.* [2].
[C] Domain structure and critical residues of influenza A virus PA protein based on Mehle and McCullers [1].

also identified in another OS-resistant virus from the same season – virus isolate A/Lisboa/15/2009. The OS-resistant virus isolate A/Lisboa/40/2008 also contained an amino acid change in PA close to a residue functionally important - I129M substitution, close to the catalytic residue K134 involved in PA endonuclease activity (PA endonuclease domain[C]; Figure 5.1E). Also of notice, the virus isolate A/Lisboa/20/2009 carried the NS1 N143D amino acid substitution located both within the nuclear export signal (NES) (residues 138-147; C-terminal effector domain[D]) and the region that interacts with the host-cell protein hGBP1 involved in the host immune response to viral infection (antiviral activity; residues 123-144) [4].

The single NA H275Y OS-resistant A(H1N1)pdm09 virus – virus isolate A/Portugal/03/2011, only contained the K52R amino acid substitution in PB1 (K13R in PB1-N40) (Table 5.1). Structurally, residue 52 is located outside of all functional domains described to date for PB1[B].

The seasonal A(H1N1) virus isolate A/Lisboa/25/2006 with decreased susceptibility to OS presented the most distinctive genome, carrying a total of 16 specific amino acid substitutions (Table 5.1). Of note, it contained the PB2 R375K and S453H substitutions in the cap-binding domain, with the former occurring adjacently to residue K376 involved in capped mRNA binding (Figure 5.1A); and the PB2 N556T and V667I substitutions in the 627 functional domain[A]. It also contained an amino acid substitution in the PB1 PB2-binding domain[B] - M744I substitution, but at some distance or far away from the residues known to be critical for stable PB2 binding (residues 695, 699, and 750; Figure 5.1C); and two further amino acid substitutions located closely to residues functionally important. Specifically, the PA K626E substitution located closely to residues involved in PB1 binding, particularly residue E623 (PA PB1-binding domain[C]; Figure 5.1F); and the F71Y substitution in the nucleocapsid protein (NP), located closely to the residues forming the putative RNA-binding site, particularly residue R175 (Figure 5.1G). The second seasonal A(H1N1) virus with decrease susceptibility to OS – virus isolate A/Lisboa/08/2008, carried the NS1 V230I amino acid substitution (Table 5.1). Residue 230 has the particularity of belonging to the target region of either PABII (residues 223-end) or PDZ domain-containing (residues 227-230) host-cell proteins, which are involved in, respectively, host pre-mRNA processing and viral pathogenesis [4,6].

[D] Domain structure and critical residues of influenza A virus NS1 protein based on Krug and García-Sastre [5].

Table 5.1 Genome-wide amino acid substitutions specific of influenza viruses resistant or with decreased susceptibility to oseltamivir and/or zanamivir (PB2, PB1, PA, NP, M and NS virus genome segments)

Influenza (sub)type	Influenza season/ Pandemic period[a]	Virus isolate	NAI susceptibility profile	GENOME SEGMENTS AND ENCODED PROTEIN(S)																									
				PB2 (segment 1)								**PB1** (segment 2)				**PA** (segment 3)						**NP** (segment 5)		**M** (segment 7)		**NS** (segment 8)			
				PB2								**PB1 (PB1-N40[b])**			**PB1-F2**	**PA (PA-N155; PA-N182[c])**			**PA-X**			**NP**		**M1**	**M2/BM2[d]**	**NS1**		**NEP (or NS2)**	
FORMER SEASONAL A(H1N1)	2005/2006	A/Lisboa/25/2006	↓**S** OS (≈2-fold)	S107N	D309G	R375K	S453H	M535L	N556T	V667I		F94S (F55S)	M744I (M705I)	-	None	I387V (I233V; I206V)	V505I (V351I; V324I)	K626E (K472E; K445E)	T216I	L236S		F71Y	S430I	None	None	None		None	
	2007/2008	A/Lisboa/08/2008	↓**S** OS (≈2-fold)			None							None		None	P332S (P178S; P515S)	-	-	None			None		None	None	V230I	-	None	
		A/Lisboa/02/2008	® OS (HRI; NA H275Y)	***K121R***	***P453S*** [e]	-	-	-	-	-			None		***H3P*** [e]		None		None			***R400K***	-	None	None	None		None	
		A/Lisboa/03/2008	® OS (HRI; NA H275Y)	***P453S*** [e]	-	-	-	-	-	-			None		***H3P*** [e]		None		None			None		None	None	None		K64R	- -
		A/Lisboa/11/2008	® OS (HRI; NA H275Y)	***K121R***	***P453S*** [e]	-	-	-	-	-		L473I/L (L434I/L) [f]	-	-	***H3P*** [e]		None		None			***R400K***	-	None	None	None		None	
		A/Lisboa/20/2008	® OS (HRI; NA H275Y)	***K121R***	***P453S*** [e]	-	-	-	-	-			None		***H3P*** [e]		None		None			***R400K***	-	None	None	None		None	
		A/Lisboa/27/2008	® OS (HRI; NA H275Y)	***P453S*** [e]	-	-	-	-	-	-			None		***H3P*** [e]	R490G (R336G; R309G)	-	-	None			- [h]		None	None	None		None	
		A/Lisboa/28/2008	® OS (HRI; NA H275Y)	***P453S*** [e]	-	-	-	-	-	-			None		***H3P*** [e]		None		None			None		None	None	None		None	
	2008/2009	A/Lisboa/29/2008	® OS (HRI; NA H275Y)																										
		A/Lisboa/39/2008	® OS (HRI; NA H275Y)																										
		A/Lisboa/40/2008	® OS (HRI; NA H275Y)	***P453S*** [e]	-	-	-	-	-	-		***N642S (N603S)*** [g]	-	-	***H3P*** [e]	I129M	-	-	I129M	-		None		None	None	None		None	
		A/Lisboa/41/2008	® OS (HRI; NA H275Y)																										
		A/Lisboa/02/2009	® OS (HRI; NA H275Y)																										
		A/Lisboa/03/2009	® OS (HRI; NA H275Y)																										
		A/Lisboa/05/2009	® OS (HRI; NA H275Y)	***P453S*** [e]	-	-	-	-	-	-		***N642S (N603S)*** [g]	-	-	***H3P*** [e]		None		None			S450N	-	None	None	D74N	-	None	
		A/Lisboa/06/2009	® OS (HRI; NA H275Y)																										
		A/Lisboa/07/2009	® OS (HRI; NA H275Y)	***P453S*** [e]	-	-	-	-	-	-		***N642S (N603S)*** [g]	-	-	***H3P*** [e]	H535P/H (H381P/H; H345P/H) [f]	M561L (M407L; M380L)	-	None			None		- [h]	- [h]	None		None	
		A/Lisboa/13/2009	® OS (HRI; NA H275Y)																										
		A/Lisboa/14/2009	® OS (HRI; NA H275Y)																										
		A/Lisboa/15/2009	® OS (HRI; NA H275Y)	T108A/T [f]	***P453S*** [e]	-	-	-	-	-		I156M (I117M)	***N642S (N603S)*** [g]	-	***H3P*** [e]	***I596V (I442V; I415V)***	-	-	None			None		None	None	None		None	
		A/Lisboa/16/2009	® OS (HRI; NA H275Y)																										
		A/Lisboa/17/2009	® OS (HRI; NA H275Y)																										
		A/Lisboa/18/2009	® OS (HRI; NA H275Y)																										
		A/Lisboa/19/2009	® OS (HRI; NA H275Y)																										
		A/Lisboa/20/2009	® OS (HRI; NA H275Y)	***P453S*** [e]	-	-	-	-	-	-		***N642S (N603S)*** [g]	-	-	***H3P*** [e]		None		None			None		None	None	C59Y	N143D	None	
		A/Lisboa/21/2009	® OS (HRI; NA H275Y)																										
		A/Lisboa/23/2009	® OS (HRI; NA H275Y)																										
		A/Lisboa/24/2009	® OS (HRI; NA H275Y)	***P453S*** [e]	-	-	-	-	-	-		F254V (F215V)	Q567E (Q528E)	***N642S (N603S)*** [g]	***H3P*** [e]	***I596V (I442V; I415V)***	-	-	None			None		None	None	Y89P	-	None	
		A/Lisboa/25/2009	® OS (HRI; NA H275Y)																										
A(H3N2)	2006/2007	A/Lisboa/01/2007	↓**S** OS (<2-fold)																										
		A/Lisboa/54/2007	↓**S** OS (<2-fold)																										
	2011/2012	A/Portugal/29/2012	↓**S** OS (≈2-fold)			None						S375G (S336G)	R602P (R563P)	-	S25F	M441T (M287T; M260T)	-	-	None			None		V97I	None	L214P	-	None	
		A/Portugal/43/2012	↓**S** OS; ZA (4-fold; NA I222V)			None							None		None	L403P (L249P; L222P)	P534S (P380S; P353S)	A618T (A464T; A437T)	None			None		None	None	None		None	
		A/Portugal/53/2012	↓**S** ZA (≈2-fold)	D152G	-	-	-	-	-	-			None		None		None		None			None		None	None	None		None	
B	2004/2005	B/Lisboa/01/2005 (YAM)	↓**S** ZA (<2-fold)			None							None				None					***V62A***	-	None	None	None		None	
		B/Lisboa/12/2005 (VIC)	↓**S** OS (≈2-fold)																										
		B/Lisboa/31/2005 (YAM)	↓**S** ZA (≈2-fold)			None							None				None					***V62A***	-	None	None	Y43H/Y [f]	-	None	
	2005/2006	B/Lisboa/05/2006 (VIC)	↓**S** OS (<2-fold)			None						***I331T***	Y729C	K741R			None					None		None	None	None		None	
		B/Lisboa/11/2006 (VIC)	↓**S** OS (<2-fold)			None						***I331T***	D553Y	-			None					- [h]		None	None	None		None	
	2006/2007	B/Lisboa/02/2007 (VIC)	↓**S** ZA (3-fold)																										
	2007/2008	B/Lisboa/34/2008 (YAM)	↓**S** OS (≈2-fold); ZA (3-fold) (NA D197N)	N16S	R686K/R [f]	-	-	-	-	-			None			I594V	-	-				K142R	-	None	None	None		V67A	- -
	2008/2009	B/Lisboa/01/2009 (VIC)	↓**S** OS; ZA (2; ≈3-fold)	V140I	-	-	-	-	-	-		M95I	-	-		S415N/S [f]	-	-				None		None	None	None		None	
	Pandemic period	B/Portugal/01/2009 (VIC)	↓**S** ZA (≈4-fold)																										
		B/Portugal/02/2009 (VIC)	↓**S** ZA (≈4-fold)			None							None				None					None		None	None	None		None	
		B/Portugal/03/2009 (YAM)	↓**S** ZA (4-fold)																										
	2010/2011	B/Portugal/01/2011 (YAM)	↓**S** ZA (3-fold)																										
		B/Portugal/02/2011 (VIC)	↓**S** OS; ZA (≈2; 4-fold)	V485I	M529I/M [f]	-	-	-	-	-			None				None					None		None	None	V140G	-	S47Y E69D N81

NOT ENCODED

NOT ENCODED

(Table 5.1 cont.)

Influenza (sub)type	Influenza season/ Pandemic period[a]	Virus isolate	NAI susceptibility profile	GENOME SEGMENTS AND ENCODED PROTEIN(S)																											
				PB2 (segment 1)								**PB1** (segment 2)				**PA** (segment 3)						**NP** (segment 5)		**M** (segment 7)		**NS** (segment 8)					
				PB2								**PB1 (PB1-N40[b])**			**PB1-F2**	**PA (PA-N155; PA-N182[c])**				**PA-X**		**NP**		**M1**	**M2/BM2[d]**	**NS1**			**NEP** (or NS2)		
A(H1N1)pdm09	Pandemic period	A/Portugal/17/2009	↓S OS (3-fold); ZA (≈2-fold) (NA I223V)	***V480I***	-	-	-	-	-	-		None			None	None				None	None		None	None	None			None		
		A/Portugal/18/2009	↓S OS (≈2-fold)	None								None			None	S296N (S142N; S115N)		-	-	None	None		None	None	P114H	-		None		
		A/Portugal/64/2009	↓S OS (≈2-fold)	None								None			None	None				None	None		None	None	None			None		
		A/Portugal/66/2009	↓S ZA (≈2-fold)	A707T	-	-	-	-	-	-		None			None	None				None	None		None	None	L43S/L[f]	-		None		
		A/Portugal/78/2009	↓S OS (≈2-fold)	R368K	-	-	-	-	-	-	P647Q (P608Q)	-	-		None	A475T (A321T; A294T)	D529N (D375N; D348N)		-	None	None		None	None	None			None		
		A/Portugal/82/2009	↓S OS (4-fold); ZA (2-fold) (NA I223V)	***V480I***	D680E	-	-	-	-	-		None			None	None				None	K400R	-	None	None	None			None		
		A/Portugal/101/2009	↓S ZA (≈2-fold)	None								None			None	V407A (V253A; V226A)		-	-	None	None		None	None	None			None		
		A/Portugal/114/2009	↓S OS (2-fold)	None							R361G (R322G)	-	-		None	None				None	L47I	-	None	None	None			None		
	2010/2011	A/Portugal/03/2011	® OS (HRI; NA H275Y - drug	None							K52R (K13R)	-	-		None	None				None	None		None	None	None			None		
		A/Portugal/07/2011	↓S OS; ZA (≈2-fold)	None								None			None	M595I (M441I; M414I)		-	-	None	None		None	None	None			None		
		A/Portugal/10/2011	↓S ZA (<2-fold)	None								None			None	None				None	None		None	None	None			None		
		A/Portugal/22/2011	↓S OS (<2-fold)	***D195N***	***R293K***	-	-	-	-	-	***G154D (G115D)***	-	-		None	L694M (L540M; L513M)		-	-	None	N473T	-	None	None	L185H	-	L28M	-	-	
	2012/2013	A/Portugal/51/2013	↓S OS; ZA (≈2; ≈3-fold)																												
		A/Portugal/52/2013	↓S OS; ZA (≈2-fold)																												
		A/Portugal/54/2013	↓S OS; ZA (2-fold)	M66I	***D195N***	***R293K***	-	-	-	-	***G154D (G115D)***	N328D (N289D)	-		None					None	None		None	None	D173N	-	T48A	-	-	
		A/Portugal/55/2013	↓S OS; ZA (≈3-fold)																												

HRI: Highly Reduced Inhibition; M1: Matrix protein; NA: Neuraminidase; NAI: Neuraminidase inhibitor; NEP: Nuclear export protein; NP: Nucleocapsid protein; NS1: Non-structural protein 1; NS2: Non-structural protein 2; OS: Oseltamivir; VIC: Victoria lineage; YAM: Yamagata lineage; ZA: Zanamivir

Symbol legend: **↓S** - Decreased Susceptibility; ® - Resistance

[a] 2009 pandemic period - period between 11th June 2009 and 9th August 2010 (WHO pandemic alert Phase 6); [b] Produced from the same reading frame as PB1 but lacks the first 39 amino acids - N-terminally truncated variant of PB1; not encoded in influenza B virus PB1 segment; [c] Produced from the same reading frame as PA but lack, respectively, the first 154 and 181 amino acids - N-terminally truncated variants of PA; not encoded in influenza B virus PA segment; [d] M segment encodes M2 protein in influenza A viruses and BM2 protein in influenza B viruses; [e] Shared by all NA H275Y OS-resistant former seasonal A(H1N1) viruses (tested + reference) - exceptionally identified against the sequence of the clade 2B OS-sensitive reference virus A/Brisbane/59/2007; [f] Mixed virus population; [g] Shared by all tested NA H275Y OS-resistant former seasonal A(H1N1) viruses from 2008/2009; also yielded by the NA H275Y OS-resistant reference virus A/England/557/2007; [h] Not successfully sequenced.

Nucleotide sequences of influenza virus genome segments were aligned by Clustal W method, edited and translated into the amino acid sequence of the protein(s) encoded in MEGA5 software. The alignment included the sequences from the viruses under study (n=14 former seasonal A(H1N1), except for NP and M (n=13); n=3 A(H3N2); n=8 B, except for NP (n=7); n=13 A(H1N1)pdm09), sequences from drug-sensitive counterparts (only A(H1N1)pdm09, n=20 (PB2, PB1, PA and NP) or 38 (M and NS) - available at the laboratory sequence database), and sequences from worldwide reference viruses available at GISAID EpiFlu™ and NCBI Influenza Virus Resource databases (n= 3 former seasonal A(H1N1), except for M (n=6); n=20 to 39 A(H3N2); n=16 to 29 B; n=5 to 24 A(H1N1)pdm09). The accession number of the sequences of the viruses circulating in Portugal, either obtained in this study or already available (drug-sensitive), shared in public-access databases can be found in Supplementary data (Table S5.1). Detailed information on the nucleotide sequences of the worldwide reference viruses is also presented in Supplementary data (see Table S5.2). Recently discovered PB2-S1, M42 and NS3 auxiliary proteins, encoded in, respectively, influenza A virus PB2, M and NS segments, were not considered as were either only express in former seasonal A(H1N1) viruses (PB2-S1) or only found in a limited number of influenza A viruses (M42 and NS3). Amino acid substitutions were identified against the virus type- or subtype-matched consensus sequence using MEGA5 (only two exceptions; see footnote e) and are indicated using influenza A or B numbering scheme. The amino acid substitutions shared by two or more viruses are highlighted in bold and italic. The influenza viruses resistant or with decreased susceptibility that were not evaluated through whole-genome sequencing, either by choice (NA H275Y OS-resistant former seasonal A(H1N1) viruses from 2008/2009 – overall resistance lead to the selection of ≈25% (6/21) of the viruses, distributed throughout the season) or by not enough volume of viral isolate to performed the analysis, are shaded in grey.

Regarding the A(H3N2) viruses with decreased susceptibility to NA inhibitors (NAIs), the NA I222V variant virus A/Portugal/43/2012 contained three amino acid substitutions in the PA PB1-binding domain[C], two of which located closely to the residues involved in PB1 binding – L403P and A618T substitutions (Figure 5.1F). And, the virus isolate A/Portugal/29/2012 yielded the NS1 L214P amino acid substitution within the region that interacts with the host-cell proteins Crk and CrkL involved in the activation of the PI3K signalling pathway (residues 212-217) [4,6].

The NA D197N influenza B variant virus B/Lisboa/34/2008 with decreased susceptibility to both NAIs carried two amino acid substitutions in PB2, located within two different functional domains. Specifically, the N16S substitution within the PB2 PB1-binding domain (residues 1-39, adapted from [A]); and the R686K substitution within the NLS domain (residues 682-770, adapted from [A]) but outside the NLS motif (residues 740-759). Other influenza B virus with dual decreased susceptibility – virus isolate B/Portugal/02/2011, contained an amino acid substitution within the PB2 cap-binding domain (residues 320-484, adapted from [A]) – V485I substitution. Structural mapping showed, however, that residue 485 is located distantly from any of the residues involved in capped mRNA binding (Figure 5.1B). The virus isolate B/Lisboa/05/2006 with decreased susceptibility to OS yielded two amino acid substitutions in the PB1 PB2-binding domain (residues 677-752, adapted from [B]) – Y729C and K741R substitutions. Both residues are located at some distance (residue 741) or far away (residue 729) from the residues known to be critical for stable PB2 binding (residues 694, 698, and 749; Figure 5.1D). Also of notice, the virus isolate B/Lisboa/31/2005 with decreased susceptibility to ZA contained the NS1 Y43H substitution within the RNA-binding domain (residues 15-93)[7], lying adjacently to residue P44 involved in the interactions with double-stranded RNA (dsRNA) (Figure 5.1I). Regarding NP genome segment, it was not possible to structurally analyse the location of the two residues on which were detected specific amino acid substitutions - residues 62 and 142. Both residues were not comprised in any of the influenza B NP structures available at the RCSB Protein Data Bank (http://www.rcsb.org/pdb).

The two NA I223V A(H1N1)pdm09 variant viruses with decreased susceptibility to OS and ZA – virus isolates A/Portugal/17/2009 and A/Portugal/82/2009, shared the PB2 V480I amino acid substitution (Table 5.1).

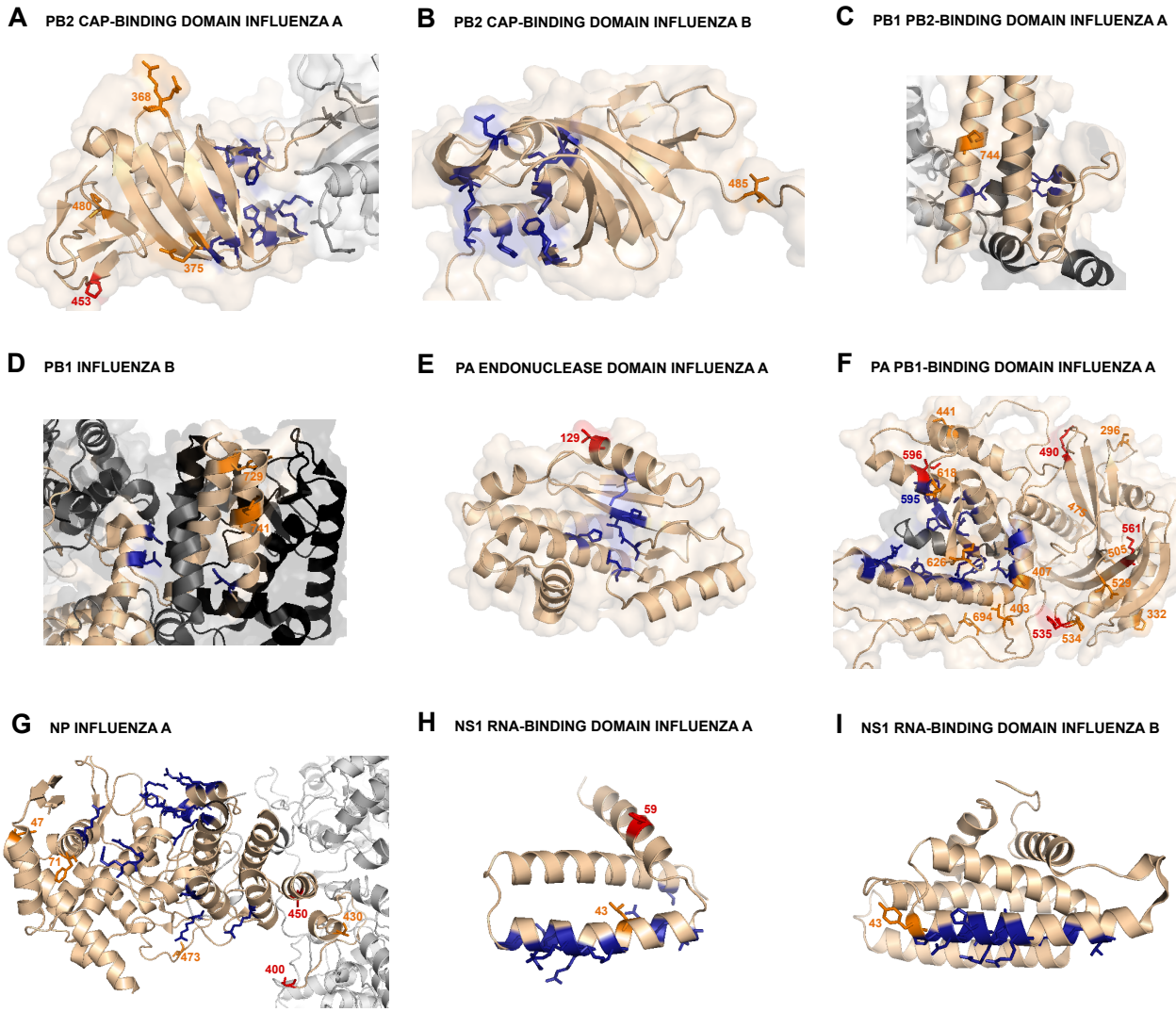

Figure 5.1 Mapping of the genome-wide residues on which were detected the amino acid substitutions specific of influenza viruses resistant or with decreased susceptibility to oseltamivir and/or zanamivir, onto the three-dimensional structure of the corresponding protein or protein domain (**A-I**).

The figures were generated in PyMOL, using protein or protein domain structure files retrieved from the RCSB Protein Data Bank (PDB) (http://www.rcsb.org/pdb). The residues on which occurred the amino acid substitutions are shown as sticks that are colour-coded according to the susceptibility profile of the virus(es) carrying the substitution: red - resistant; orange – with decreased susceptibility. When viruses from both categories carried amino acid substitutions at the same residue, this was coloured in red (prevalence of resistant profile). The residues known to be functionally important are shown as dark blue sticks, maintaining the colour assigned to functional relevance when also associated with the occurrence of an amino acid substitution. The residues are numbered according to the influenza type specific numbering scheme (influenza A or B). **(A)** Structure of the PB2 cap-binding domain from an influenza A virus (PDB ID: 4U6O; residues 318-483). The PDB file also includes the cap-binding domain of a second PB2 monomer that is shown in light grey. **(B)** Structure of the PB2 cap-binding domain from the influenza B virus B/Lee/1940 (PDB ID: 5EF9; residues 420-484). **Panel A and B**: the residues known to be functionally relevant are those involved in the binding to the 5´cap of host mRNAs (cap-binding residues; based on Mehle and McCullers [1]). **(C)** Structure of the PB1 PB2-binding domain from the former seasonal A(H1N1) virus A/Puerto Rico/08/1934 (PDB ID: 3A1G; residues 678-757). The PDB file also includes the PB1-binding domain of PB2 (shown in dark grey) and the PB1-PB2 interface of a second monomer (shown in light grey). **(D)** Structure of the PB1 protein from the influenza B virus B/Memphis/13/2003 (PDB ID: 5FMZ). The PDB file also includes the PB2 (shown in dark grey) and PA (shown in black) proteins - RNA polymerase complex.

(Footnotes Figure 5.1 cont.)

Panel C and D: the PB1 residues indicated as functionally important are those known to be critical for stable binding to PB2 (residues 695, 699, 750 (influenza A numbering); based on Mehle and McCullers [1]). **(E)** Structure of the PA endonuclease domain from the reference A(H1N1)pdm09 virus A/California/04/2009 (PDB ID: 5DES; residues 1-196). Functionally important residues include those coordinating the binding to the 2 Mn^{2+} metal ions (metal-dependent endonuclease) and residue K143 that interacts directly with host mRNAs during the cleavage of its 5' cap (cap-snatching endonuclease; residue exceptionally coloured in light purple) [1]. **(F)** Structure of the PA PB1-binding domain from the former seasonal A(H1N1) virus A/Puerto Rico/08/1934 (PDB ID: 2ZNL; residues 239-716). The PDB file also includes the PA-binding domain of PB1 (shown in dark grey) – PA-PB1 interface. PA residue 387 on which was detected the I387V amino acid substitution could not be indicated as is missing in the structure file used. The PA residues known to be functionally important are those involved in the binding to PB1 (based on Mehle and McCullers [1]). **(G)** Structure of the nucleocapsid protein (NP) from the former seasonal A(H1N1) virus A/WSN/1933 (PDB ID: 2IQH). The PDB file also describes the two other monomers comprising the crystallized NP (homotrimer), shown in light grey. Functionally important residues include those comprising the putative RNA-binding site (based on Ye *et al.* [8]). **(H)** Structure of the NS1 RNA-binding domain from the A(H3N2) virus A/Udorn/307/1972 (PDB ID: 1AIL; residues 1-73). **(I)** Structure of the NS1 RNA-binding domain from the influenza B virus B/Lee/40 (PDB ID: 1XEQ; residues 15-93). **Panel H and I**: the residues indicated as functionally important are those mediating the interaction with dsRNA (based on Krug and García-Sastre [5] and Hale *et al.* [6]).

Structurally, residue 480 belongs to the PB2 cap-binding domain but is located distantly from the residues involved in capped mRNA binding, as shown in Figure 5.1A. A/Portugal/82/2009 contained a second specific PB2 amino acid substitution in the NLS domain[A], located outside the NLS motif (residues 736-755) – D680E substitution. Additional PB2 amino acid substitutions located within functional domains included: PB2 R368K substitution in A/Portugal/78/2009 (cap-binding domain, but located distantly from cap-binding residues (Figure 5.1A)); and PB2 A707T substitution in A/Portugal/66/2009 (NLS domain, outside NLS motif). This latter also contained the NS1 L43S substitution at the RNA-binding domain[D], located closely to the residues mediating the interactions with dsRNA (Figure 5.1H). A(H1N1)pdm09 virus isolates A/Portugal/101/2009 and A/Portugal/07/2011 with decreased susceptibility to OS and/or ZA carried an amino acid substitution in the PA PB1-binding domain - V407A and M595I substitutions, respectively. Structural mapping showed that residue 407 lies adjacently to residue Q408 involved in PB1 binding (Figure 5.1F), while residue 595 is directly involved in the binding to this other polymerase subunit. Also of note, the virus isolate A/Portugal/114/2009 with decreased susceptibility to OS contained the NP L47I amino acid substitution located within the sequence of the NES 1 (residues 29-49); and the virus isolates A/Portugal/22/2011 and A/Portugal/54/2013 with decreased susceptibility to OS and/or ZA contained an amino acid change within the region of the NS1 that interacts with the host-cell factor CPSF30 involved in pre-mRNA processing (residues 103, 106, and 144-188) [4,6] – L185H and D173N substitutions, respectively.

5.2 DISCUSSION

Genetic characterization of influenza viruses resistant or with decreased susceptibility to NAIs usually stays limited to the two genome segments in which can occur amino acid substitutions affecting virus susceptibility - NA and HA segments [9]. The emergence and worldwide spread of a fit and transmissible drug-resistant variant in late 2007 (NA H275Y seasonal A(H1N1)) showed, however, the importance of expanding genetic characterization to all other 6 segments of influenza virus genome (PB2, PB1, PA, NP, M and NS segments). Amino acid changes at all genome segments can affect the overall virus fitness[E] and compensate for the expected fitness deficit of drug-resistant variants or even result in an enhanced virus fitness compared to drug-sensitive counterparts. Transmissible drug resistance poses a serious threat to public health, as it could render NAIs useless against circulating human influenza viruses and left us without any options for antiviral therapy. Elucidating the molecular mechanisms underlying fitness gains in drug-resistant viruses is therefore a priority in influenza.

NA H275Y OS-resistant seasonal A(H1N1) viruses shared the PB2 P453S and PB1-F2 H3P amino acid substitutions, with those from 2008/2009 further sharing the PB1 N642S amino acid substitution. However, the PB1-F2 protein of seasonal A(H1N1) viruses was present in a C-terminally truncated non-functional form and structural analysis of either PB2 P453S or PB1 N642S substitutions suggested no role in the enhanced viral fitness of these drug-resistant viruses. PB2 P453S occurred within the cap-binding functional domain but distantly from the residues directly involved in the binding to the host mRNAs; while PB1 N642S occurred outside of all known functional domains. Notwithstanding, it has to be taken into consideration that, contrary to the other two polymerase subunits (PB2 and PA), only limited structural information is available for PB1 [1]. Both PB2 P453S and PB1 N642S substitutions were identified in a previous genome-wide study targeting seasonal A(H1N1) viruses circulating in Taiwan from 2005 to 2009, distinguishing clade 2B OS-resistant and OS-sensitive viruses [12]. But, no information is available about its effect on overall virus fitness. Further *in vitro* and *in vivo* studies are still required to clarify the role of both amino acid substitutions in the enhanced viral fitness of NA H275Y OS-resistant viruses. Particularly of PB1 N642S,

[E] Considered as the ability of the virus to replicate efficiently (replicative fitness) and transmit readily between hosts (transmission fitness) [10]. This takes into consideration both standard (*"the capacity of a virus to produce infectious progeny in a given environment"*) and Darwinian (*"the amount of genetic material passed on to the next generation"*) definitions of overall fitness [11].

which was the only amino acid substitution shared by the latter drug-resistant viruses (2008/2009) that spread over their sensitive counterparts and fixed NA H275Y in A(H1N1) virus population (100% resistance).

Amino acid changes in or close to residues known to be functionally important were detected individually in either NA H275Y OS-resistant seasonal A(H1N1) viruses from 2008/2009 or influenza viruses with decreased susceptibility to OS and/or ZA from all different types and subtypes. Based on its structural location, these amino acid substitutions may play a role in the overall fitness of the virus. In fact, PB2 V667I substitution is known to be associated with an enhanced transmission to humans (molecular marker of enhanced transmission) [13], but no information was found about any of the other substitutions in the literature. It is also important to note that previous genetic characterization of HA gene (genotypic susceptibility testing; Chapter 4) identified the potential fitness-enhancing mutation HA N156K in the A(H1N1)pdm09 virus A/Portugal/07/2011 with decreased susceptibility to both NAIs.

The NA H275Y OS-resistant A(H1N1)pdm09 virus isolate A/Portugal/03/2011 carried a single amino acid substitution in PB1 (PB1 K52R) that, based on its structural location outside of all known functional domains, had probably no impact on viral fitness. Nonetheless, only limited structural information is available for PB1, as above-mentioned.

Previous *in vitro* studies showed that the PB2 T108A, D195N and M535L amino acid substitutions found in influenza N1 NA viruses resistant or with decrease susceptibility to OS and/or ZA (A/Lisboa/15/2009 (seasonal N1), A/Portugal/22/2011 and A/Portugal/54/2013 (N1pdm09), and A/Lisboa/25/2006 (seasonal N1), respectively), increase the activity of the viral ribonucleoprotein (RNP) complex [14-16]. Considering that the RNP complex mediates both transcription and replication of viral RNA (vRNA) genome [1], the enhanced activity associated with the presence of such amino acid substitutions may be linked to an increase in viral transcription and/or replication efficiency. The T48A amino acid substitution additionally found in the nuclear export protein (NEP) of A/Portugal/54/2013 is know to increase the binding to the host-cell protein CRM1 that mediates the export of viral RNP complexes from the nucleus (nuclear transport receptor) [17]. This increased interaction with CMR1 may result in enhanced

nuclear export of viral RNP complexes and consequently enhanced viral replication. The NS1 D74N amino acid substitution identified in an additional OS-resistant seasonal N1 virus – virus isolate A/Lisboa/05/2009, is known to significantly increase viral replication *in vitro* in influenza A(H1N1)pdm09 and A(H5N1) backgrounds [18]. The substitution was studied in more detailed in A(H5N1) avian viruses, in which showed to alter the viral RNP complex export dynamics and increase its activity, enhance viral genome and protein synthesis, and improve replication and transcription activities. Also, given its location in the linker region, it was proposed to alter the structural flexibility of NS1, affecting how the specific protein-RNA or protein-protein interactions occur in either RNA-binding or effector functional domains [18].

5.3 CONCLUSIONS

This study enabled the complete characterization of the genome of influenza viruses resistant or with decreased susceptibility to OS and/or ZA. It contributed at finding potential determinants of viral fitness in other segments of influenza virus genome beyond NA and HA; and at elucidating the potential lack of role of these other segments in the enhanced viral fitness of NA H275Y OS-resistant seasonal A(H1N1) viruses that spread worldwide. In a broader sense, it also contributed at a better understanding of the genetic changes underlying the evolution of influenza virus genomes.

BOX 5.2 - MAIN FINDINGS

- Structural location of PB2 P453S and PB1 N642S amino acid substitutions shared by NA H275Y OS-resistant seasonal A(H1N1) viruses suggests no role in their fitness advantage over OS-sensitive counterparts.
- Additional PB1-F2 H3P amino acid substitution specific of NA H275Y OS-resistant seasonal A(H1N1) viruses occurred in a C-terminally truncated non-functional protein.
- NA H275Y OS-resistant A(H1N1)pdm09 virus with only a single amino acid substitution in PB1, outside of all known functional domains (PB1 K52R).
- PB2 T108A, D195N, M535L and V667I amino acid substitutions, as well as NEP T48A and NS1 D74N amino acid substitutions, detected in A(H1N1) viruses from either former or current circulating subtypes resistant or with decreased susceptibility to OS and/or ZA, are known to or may play a role in enhance overall virus fitness.

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SUPPLEMENTARY DATA

Table S5.1 List of the accession number of the genome-wide nucleotide sequences of influenza viruses circulating in Portugal used in this study and shared in public-access databases.

Influenza virus subtype	2009 pandemic period[a] / Influenza season	Sequence database	Accession number	Segment	Virus isolate	NAI susceptibility profile
A(H1N1)pdm09	Pandemic period	GISAID EPIFLU™	EPI500694	PB1	A/Portugal/17/2009	**↓S** OS (3-fold); ZA (≈2-fold) (NA I223V)
			EPI500725	PB1	A/Portugal/52/2009	Sensitive
			EPI500750	PB1	A/Portugal/01/2010	Sensitive
			EPI500753	PB1	A/Portugal/02/2010	Sensitive
	2010/2011		EPI500762	PB1	A/Portugal/21/2011	Sensitive
			EPI500767	PB1	A/Portugal/28/2011	Sensitive

NA: Neuraminidase; NAI: Neuraminidase inhibitor; OS: Oseltamivir; ZA: Zanamivir

Symbol legend: **↓S** - Decreased Susceptibility

[a] Period from 11th June 2009 to 9th August 2010

GISAID EpiFlu™ database: http://platform.gisaid.org.

The sequences were either generated in this study (A/Portugal/17/2009) or were already available at the laboratory sequence database (drug-sensitive viruses), having been obtained in the context of the study on the evolutionary dynamics of the newly emerging A(H1N1) pandemic virus.

I acknowledge the authors, originating and submitting laboratories of the genome-wide nucleotide sequences of worldwide reference influenza viruses retrieved from GISAID EpiFlu™ and NCBI Influenza Virus Resource databases that were used in this study.

Table S5.2 Detailed information on the genome-wide nucleotide sequences of worldwide reference influenza viruses used in this study (PB2, PB1, PA, NP, M and NS segments), retrieved from GISAID EpiFlu™ and NCBI Influenza Virus Resource databases.

Influenza (sub)type	Segment ID	Segment	Country	Collection date (year-month-day)	Reference virus	Originating Lab	Submitting Lab	Authors
Former seasonal A(H1N1)	EPI249527	PB2	Australia	2007-Jan-01	***A/Brisbane/59/2007***	-	Other Database Import	Webby,R.; Webster,R.G.; Govorkova,E.; Duan,S.
	EPI249528	PB1	Australia	2007-Jan-01	***A/Brisbane/59/2007***	-	Other Database Import	Webby,R.; Webster,R.G.; Govorkova,E.; Duan,S.
	EPI249529	PA	Australia	2007-Jan-01	***A/Brisbane/59/2007***	-	Other Database Import	Webby,R.; Webster,R.G.; Govorkova,E.; Duan,S.
	EPI249531	NP	Australia	2007-Jan-01	***A/Brisbane/59/2007***	-	Other Database Import	Webby,R.; Webster,R.G.; Govorkova,E.; Duan,S.
	EPI249533	M	Australia	2007-Jan-01	***A/Brisbane/59/2007***	-	Other Database Import	Webby,R.; Webster,R.G.; Govorkova,E.; Duan,S.
	EPI249534	NS	Australia	2007-Jan-01	***A/Brisbane/59/2007***	-	Other Database Import	Webby,R.; Webster,R.G.; Govorkova,E.; Duan,S.
	EPI168128	PB1	United Kingdom	2007-Nov-15	A/England/557/2007	-	Other Database Import	-
	EPI168130	PA	United Kingdom	2007-Nov-15	A/England/557/2007	-	Other Database Import	-
	EPI168131	PB2	United Kingdom	2007-Nov-15	A/England/557/2007	-	Other Database Import	-
	EPI168132	NP	United Kingdom	2007-Nov-15	A/England/557/2007	-	Other Database Import	-
	EPI168133	M	United Kingdom	2007-Nov-15	A/England/557/2007	-	Other Database Import	-
	EPI168134	NS	United Kingdom	2007-Nov-15	A/England/557/2007	-	Other Database Import	-
	EPI155236	M	Japan	2006-Jan-17	A/Fukushima/141/2006	-	Centers for Disease Control and Prevention	-
	EPI159399	PB2	New Caledonia	1999-Jan-01	***A/New Caledonia/20/1999***	-	Other Database Import	-
	EPI159398	PB1	New Caledonia	1999-Jan-01	***A/New Caledonia/20/1999***	-	Other Database Import	-
	EPI159397	PA	New Caledonia	1999-Jan-01	***A/New Caledonia/20/1999***	-	Other Database Import	-
	EPI159395	NP	New Caledonia	1999-Jan-01	***A/New Caledonia/20/1999***	-	Other Database Import	-
	EPI159393	M	New Caledonia	1999-Jan-01	***A/New Caledonia/20/1999***	-	Other Database Import	-
	EPI159396	NS	New Caledonia	1999-Jan-01	***A/New Caledonia/20/1999***	-	Other Database Import	-
	EPI152158	M	Solomon Islands	2006-Aug-21	***A/Solomon Islands/03/2006***	-	Other Database Import	-
	EPI189429	M	Russian Federation	2008-Feb-13	A/St. Petersburg/12/2008	-	Centers for Disease Control and Prevention	-
A(H3N2)	EPI349372	M	United States	2011-Dec-07	A/Alabama/04/2011	ADPH Bureau of Clinical Laboratories	Centers for Disease Control and Prevention	-
	EPI341641	PB2	United States	2010-Jul-13	A/Alabama/05/2010	U.S. Air Force School of Aerospace Medicine	Centers for Disease Control and Prevention	-
	EPI341642	PB1	United States	2010-Jul-13	A/Alabama/05/2010	U.S. Air Force School of Aerospace Medicine	Centers for Disease Control and Prevention	-
	EPI341640	PA	United States	2010-Jul-13	A/Alabama/05/2010	U.S. Air Force School of Aerospace Medicine	Centers for Disease Control and Prevention	-
	EPI341638	NP	United States	2010-Jul-13	A/Alabama/05/2010	U.S. Air Force School of Aerospace Medicine	Centers for Disease Control and Prevention	-
	EPI278803	M	United States	2010-Jul-13	A/Alabama/05/2010	U.S. Air Force School of Aerospace Medicine	Centers for Disease Control and Prevention	-
	EPI341639	NS	United States	2010-Jul-13	A/Alabama/05/2010	U.S. Air Force School of Aerospace Medicine	Centers for Disease Control and Prevention	-
	EPI353310	PB2	Australia	2007-Feb-06	***A/Brisbane/10/2007***	Queensland Health Scientific Services	WHO Collaborating Centre for Reference and Research on Influenza	Iannello,P; Komadina,N
	EPI353309	PB1	Australia	2007-Feb-06	***A/Brisbane/10/2007***	Queensland Health Scientific Services	WHO Collaborating Centre for Reference and Research on Influenza	Iannello,P; Komadina,N
	EPI353308	PA	Australia	2007-Feb-06	***A/Brisbane/10/2007***	Queensland Health Scientific Services	WHO Collaborating Centre for Reference and Research on Influenza	Iannello,P; Komadina,N
	EPI353307	NP	Australia	2007-Feb-06	***A/Brisbane/10/2007***	Queensland Health Scientific Services	WHO Collaborating Centre for Reference and Research on Influenza	Iannello,P; Komadina,N
	EPI353305	M	Australia	2007-Feb-06	***A/Brisbane/10/2007***	Queensland Health Scientific Services	WHO Collaborating Centre for Reference and Research on Influenza	Iannello,P; Komadina,N
	EPI353306	NS	Australia	2007-Feb-06	***A/Brisbane/10/2007***	Queensland Health Scientific Services	WHO Collaborating Centre for Reference and Research on Influenza	Iannello,P; Komadina,N
	EPI175105	M	Australia	2008-Jun-23	A/Brisbane/24/2008	-	Centers for Disease Control and Prevention	-
	EPI228932	PB2	United States	2004-Jan-01	***A/California/07/2004***	-	Other Database Import	Bragstad,K.; Nielsen,L.P.; Fomsgaard,A.
	EPI228943	PA	United States	2004-Jan-01	***A/California/07/2004***	-	Other Database Import	Bragstad,K.; Nielsen,L.P.; Fomsgaard,A.
	EPI228945	M	United States	2004-Jan-01	***A/California/07/2004***	-	Other Database Import	Bragstad,K.; Nielsen,L.P.; Fomsgaard,A.
	EPI228949	NS	United States	2004-Jan-01	***A/California/07/2004***	-	Other Database Import	Bragstad,K.; Nielsen,L.P.; Fomsgaard,A.
	EPI302281	M	United States	2010-Dec-08	A/California/28/2010	California Department of Health Services	Centers for Disease Control and Prevention	-
	EPI500570	M	Senegal	2012-Oct-11	A/Dakar/15/2012	Institut Pasteur de Dakar	National Institute for Medical Research	-
	EPI490084	PB2	United States	2012-Nov-12	A/Delaware/15/2012	Delaware Public Health Lab	Centers for Disease Control and Prevention	-
	EPI490085	PB1	United States	2012-Nov-12	A/Delaware/15/2012	Delaware Public Health Lab	Centers for Disease Control and Prevention	-
	EPI490083	PA	United States	2012-Nov-12	A/Delaware/15/2012	Delaware Public Health Lab	Centers for Disease Control and Prevention	-
	EPI417235	M	United States	2012-Nov-12	A/Delaware/15/2012	Delaware Public Health Lab	Centers for Disease Control and Prevention	-
	EPI490081	NP	United States	2012-Nov-12	A/Delaware/15/2012	Delaware Public Health Lab	Centers for Disease Control and Prevention	-
	EPI490082	NS	United States	2012-Nov-12	A/Delaware/15/2012	Delaware Public Health Lab	Centers for Disease Control and Prevention	-

(Table S5.2 cont.)

Influenza (sub)type	Segment ID	Segment	Country	Collection date (year-month-day)	Reference virus	Originating Lab	Submitting Lab	Authors
	EPI508354	M	United Kingdom	2010-Nov-26	A/England/4820166/2010	Health Protection Agency	National Institute for Medical Research	-
	EPI358781	PB2	China	2002-Jan-01	***A/Fujian/411/2002***	-	Other Database Import	Galiano,M.; Johnson,B.F.; Myers,R.; Ellis,J.; Daniels,R.; *et al.*
	EPI358782	PB1	China	2002-Jan-01	***A/Fujian/411/2002***	-	Other Database Import	Galiano,M.; Johnson,B.F.; Myers,R.; Ellis,J.; Daniels,R.; *et al.*
	EPI358783	PA	China	2002-Jan-01	***A/Fujian/411/2002***	-	Other Database Import	Galiano,M.; Johnson,B.F.; Myers,R.; Ellis,J.; Daniels,R.; *et al.*
	EPI358785	NP	China	2002-Jan-01	***A/Fujian/411/2002***	-	Other Database Import	Galiano,M.; Johnson,B.F.; Myers,R.; Ellis,J.; Daniels,R.; *et al.*
	EPI358787	M	China	2002-Jan-01	***A/Fujian/411/2002***	-	Other Database Import	Galiano,M.; Johnson,B.F.; Myers,R.; Ellis,J.; Daniels,R.; *et al.*
	EPI358788	NS	China	2002-Jan-01	***A/Fujian/411/2002***	-	Other Database Import	Galiano,M.; Johnson,B.F.; Myers,R.; Ellis,J.; Daniels,R.; *et al.*
	EPI342128	M	United States	2009-Mar-30	A/Hawaii/07/2009	State of Hawaii Department of Health	Centers for Disease Control and Prevention	
	EPI142344	PB2	Japan	2005-Oct-24	A/Hiroshima/52/2005	-	Other Database Import	Krammer,F.; Grabherr,R.
	EPI142345	PB1	Japan	2005-Oct-24	A/Hiroshima/52/2005	-	Other Database Import	Krammer,F.; Grabherr,R.
	EPI142346	PA	Japan	2005-Oct-24	A/Hiroshima/52/2005	-	Other Database Import	Krammer,F.; Grabherr,R.
	EPI142347	NP	Japan	2005-Oct-24	A/Hiroshima/52/2005	-	Other Database Import	Krammer,F.; Grabherr,R.
	EPI142349	M	Japan	2005-Oct-24	A/Hiroshima/52/2005	-	Other Database Import	Krammer,F.; Grabherr,R.
	EPI142348	NS	Japan	2005-Oct-24	A/Hiroshima/52/2005	-	Other Database Import	Krammer,F.; Grabherr,R.
	EPI353509	PB2	Hong Kong (SAR)	2009-Apr-07	A/Hong Kong/2000/2009	Government Virus Unit	Centers for Disease Control and Prevention	
	EPI353510	PB1	Hong Kong (SAR)	2009-Apr-07	A/Hong Kong/2000/2009	Government Virus Unit	Centers for Disease Control and Prevention	
	EPI353508	PA	Hong Kong (SAR)	2009-Apr-07	A/Hong Kong/2000/2009	Government Virus Unit	Centers for Disease Control and Prevention	
	EPI353505	NP	Hong Kong (SAR)	2009-Apr-07	A/Hong Kong/2000/2009	Government Virus Unit	Centers for Disease Control and Prevention	
	EPI353507	M	Hong Kong (SAR)	2009-Apr-07	A/Hong Kong/2000/2009	Government Virus Unit	Centers for Disease Control and Prevention	
	EPI353506	NS	Hong Kong (SAR)	2009-Apr-07	A/Hong Kong/2000/2009	Government Virus Unit	Centers for Disease Control and Prevention	
	EPI335920	PB2	United States	2010-Dec-30	A/Iowa/19/2010	Iowa State Hygienic Laboratory	Centers for Disease Control and Prevention	
	EPI335921	PB1	United States	2010-Dec-30	A/Iowa/19/2010	Iowa State Hygienic Laboratory	Centers for Disease Control and Prevention	
	EPI335919	PA	United States	2010-Dec-30	A/Iowa/19/2010	Iowa State Hygienic Laboratory	Centers for Disease Control and Prevention	
	EPI335916	NP	United States	2010-Dec-30	A/Iowa/19/2010	Iowa State Hygienic Laboratory	Centers for Disease Control and Prevention	
	EPI335918	M	United States	2010-Dec-30	A/Iowa/19/2010	Iowa State Hygienic Laboratory	Centers for Disease Control and Prevention	
	EPI335917	NS	United States	2010-Dec-30	A/Iowa/19/2010	Iowa State Hygienic Laboratory	Centers for Disease Control and Prevention	
	EPI232566	M	Kenya	2009-Sep-15	A/Kenya/4408/2009	CDC-Kenya	Centers for Disease Control and Prevention	
	EPI417175	M	United States	2012-Nov-13	A/Maryland/43/2012	Maryland Department of Health and Mental Hygiene	Centers for Disease Control and Prevention	
A(H3N2)	EPI390187	PB1	Nepal	2006-Aug-01	A/Nepal/921/2006	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI390188	PB2	Nepal	2006-Aug-01	A/Nepal/921/2006	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI390186	PA	Nepal	2006-Aug-01	A/Nepal/921/2006	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI390184	NP	Nepal	2006-Aug-01	A/Nepal/921/2006	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI390182	M	Nepal	2006-Aug-01	A/Nepal/921/2006	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI390185	NS	Nepal	2006-Aug-01	A/Nepal/921/2006	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI381978	PB2	United States	2004-Jan-01	A/New York/55/2004	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI381977	PB1	United States	2004-Jan-01	A/New York/55/2004	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI381976	PA	United States	2004-Jan-01	A/New York/55/2004	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI381975	NP	United States	2004-Jan-01	A/New York/55/2004	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI381972	M	United States	2004-Jan-01	A/New York/55/2004	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI381974	NS	United States	2004-Jan-01	A/New York/55/2004	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI508321	M	Norway	2010-Dec-03	A/Norway/1330/2010	WHO National Influenza Centre	National Institute for Medical Research	
	EPI397576	PB2	United States	2012-Mar-08	A/Ohio/02/2012	WHO Collaborating Centre for Reference and Research on Influenza	Centers for Disease Control and Prevention	
	EPI397577	PB1	United States	2012-Mar-08	A/Ohio/02/2012	WHO Collaborating Centre for Reference and Research on Influenza	Centers for Disease Control and Prevention	
	EPI397638	PA	United States	2012-Mar-08	A/Ohio/02/2012	WHO Collaborating Centre for Reference and Research on Influenza	Centers for Disease Control and Prevention	
	EPI397637	NP	United States	2012-Mar-08	A/Ohio/02/2012	WHO Collaborating Centre for Reference and Research on Influenza	Centers for Disease Control and Prevention	
	EPI384819	M	United States	2012-Mar-08	A/Ohio/02/2012	WHO Collaborating Centre for Reference and Research on Influenza	Centers for Disease Control and Prevention	
	EPI397575	NS	United States	2012-Mar-08	A/Ohio/02/2012	WHO Collaborating Centre for Reference and Research on Influenza	Centers for Disease Control and Prevention	
	EPI500625	M	France	2012-Oct-29	A/Paris/1651/2012	Institut Pasteur	National Institute for Medical Research	
	EPI390180	PB2	Australia	2010-Aug-01	A/Perth/10/2010	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI390179	PB1	Australia	2010-Aug-01	A/Perth/10/2010	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI390178	PA	Australia	2010-Aug-01	A/Perth/10/2010	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI390120	NP	Australia	2010-Aug-01	A/Perth/10/2010	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI390118	M	Australia	2010-Aug-01	A/Perth/10/2010	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI390121	NS	Australia	2010-Aug-01	A/Perth/10/2010	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI272741	PB2	Australia	2009-Apr-07	***A/Perth/16/2009***	Pathwest QE II Medical Centre	WHO Collaborating Centre for Reference and Research on Influenza	Deng,Y.M.; Iannello,P; Erneste,J; Komadina,N.
	EPI272742	PB1	Australia	2009-Apr-07	***A/Perth/16/2009***	Pathwest QE II Medical Centre	WHO Collaborating Centre for Reference and Research on Influenza	Deng,Y.M.; Iannello,P; Erneste,J; Komadina,N.
	EPI272743	PA	Australia	2009-Apr-07	***A/Perth/16/2009***	Pathwest QE II Medical Centre	WHO Collaborating Centre for Reference and Research on Influenza	Deng,Y.M.; Iannello,P; Erneste,J; Komadina,N.
	EPI272744	NP	Australia	2009-Apr-07	***A/Perth/16/2009***	Pathwest QE II Medical Centre	WHO Collaborating Centre for Reference and Research on Influenza	Deng,Y.M.; Iannello,P; Erneste,J; Komadina,N.
	EPI272746	M	Australia	2009-Apr-07	***A/Perth/16/2009***	Pathwest QE II Medical Centre	WHO Collaborating Centre for Reference and Research on Influenza	Deng,Y.M.; Iannello,P; Erneste,J; Komadina,N.
	EPI272745	NS	Australia	2009-Apr-07	***A/Perth/16/2009***	Pathwest QE II Medical Centre	WHO Collaborating Centre for Reference and Research on Influenza	Deng,Y.M.; Iannello,P; Erneste,J; Komadina,N.

(Table S4.2 cont.)

Influenza (sub)type	Segment ID	Segment	Country	Collection date (year-month-day)	Reference virus	Originating Lab	Submitting Lab	Authors
A(H3N2)	EPI390384	PB2	United States	2010-Aug-01	A/Rhode Island/01/2010	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI390383	PB1	United States	2010-Aug-01	A/Rhode Island/01/2010	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI390382	PA	United States	2010-Aug-01	A/Rhode Island/01/2010	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI390380	NP	United States	2010-Aug-01	A/Rhode Island/01/2010	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI390378	M	United States	2010-Aug-01	A/Rhode Island/01/2010	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI390381	NS	United States	2010-Aug-01	A/Rhode Island/01/2010	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI507964	M	Sweden	2011-Mar-28	A/Stockholm/18/2011	Swedish Institute for Infectious Disease Control	National Institute for Medical Research	
	EPI238692	M	Sweden	2009-Aug-01	A/Stockholm/90/2009	Swedish Institute for Infectious Disease Control	Swedish Institute for Infectious Disease Control	
	EPI556813	PB2	United States	2012-Apr-15	***A/Texas/50/2012***	Texas Department of State Health Services-Laboratory Services	Centers for Disease Control and Prevention	
	EPI556814	PB1	United States	2012-Apr-15	***A/Texas/50/2012***	Texas Department of State Health Services-Laboratory Services	Centers for Disease Control and Prevention	
	EPI556812	PA	United States	2012-Apr-15	***A/Texas/50/2012***	Texas Department of State Health Services-Laboratory Services	Centers for Disease Control and Prevention	
	EPI556809	NP	United States	2012-Apr-15	***A/Texas/50/2012***	Texas Department of State Health Services-Laboratory Services	Centers for Disease Control and Prevention	
	EPI556811	M	United States	2012-Apr-15	***A/Texas/50/2012***	Texas Department of State Health Services-Laboratory Services	Centers for Disease Control and Prevention	
	EPI556810	NS	United States	2012-Apr-15	***A/Texas/50/2012***	Texas Department of State Health Services-Laboratory Services	Centers for Disease Control and Prevention	
	EPI152549	M	Uruguay	2007-Jan-01	A/Uruguay/716/2007	-	Centers for Disease Control and Prevention	
	EPI232627	M	United States	2009-Sep-30	A/Vermont/22/2009	Vermont Department of Health Laboratory	Centers for Disease Control and Prevention	
	EPI381911	NP	Australia	2009-Aug-01	A/Victoria/210/2009	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI381909	M	Australia	2009-Aug-01	A/Victoria/210/2009	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI381912	NS	Australia	2009-Aug-01	A/Victoria/210/2009	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI417234	PB2	Australia	2011-Oct-24	***A/Victoria/361/2011***	WHO Collaborating Centre for Reference and Research on Influenza	Centers for Disease Control and Prevention	
	EPI418016	PB1	Australia	2011-Oct-24	***A/Victoria/361/2011***	WHO Collaborating Centre for Reference and Research on Influenza	Centers for Disease Control and Prevention	
	EPI418015	PA	Australia	2011-Oct-24	***A/Victoria/361/2011***	WHO Collaborating Centre for Reference and Research on Influenza	Centers for Disease Control and Prevention	
	EPI440094	NP	Australia	2011-Oct-24	***A/Victoria/361/2011***	WHO Collaborating Centre for Reference and Research on Influenza	Centers for Disease Control and Prevention	
	EPI417233	M	Australia	2011-Oct-24	***A/Victoria/361/2011***	WHO Collaborating Centre for Reference and Research on Influenza	Centers for Disease Control and Prevention	
	EPI417232	NS	Australia	2011-Oct-24	***A/Victoria/361/2011***	WHO Collaborating Centre for Reference and Research on Influenza	Centers for Disease Control and Prevention	
	EPI40997	PB2	New Zealand	2005-Sep-13	A/Waikato/12/2005	-	Other Database Import	
	EPI40994	PB1	New Zealand	2005-Sep-13	A/Waikato/12/2005	-	Other Database Import	
	EPI40992	PA	New Zealand	2005-Sep-13	A/Waikato/12/2005	-	Other Database Import	
	EPI40987	NP	New Zealand	2005-Sep-13	A/Waikato/12/2005	-	Other Database Import	
	EPI40982	M	New Zealand	2005-Sep-13	A/Waikato/12/2005	-	Other Database Import	
	EPI40989	NS	New Zealand	2005-Sep-13	A/Waikato/12/2005	-	Other Database Import	
	EPI185936	M	United States	2009-Apr-06	A/Washington/15/2009	Washington State Public Health Laboratory	Centers for Disease Control and Prevention	
	EPI416179	M	United States	2012-Dec-05	A/Washington/60/2012	Spokane Regional Health District	Centers for Disease Control and Prevention	
	EPI390116	PB2	New Zealand	2004-Aug-01	A/Wellington/01/2004	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI390115	PB1	New Zealand	2004-Aug-01	A/Wellington/01/2004	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI390114	PA	New Zealand	2004-Aug-01	A/Wellington/01/2004	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI390112	NP	New Zealand	2004-Aug-01	A/Wellington/01/2004	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI390110	M	New Zealand	2004-Aug-01	A/Wellington/01/2004	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI390113	NS	New Zealand	2004-Aug-01	A/Wellington/01/2004	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI390196	PB2	United States	2007-Aug-01	A/Wisconsin/03/2007	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI390195	PB1	United States	2007-Aug-01	A/Wisconsin/03/2007	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI390194	PA	United States	2007-Aug-01	A/Wisconsin/03/2007	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI390192	NP	United States	2007-Aug-01	A/Wisconsin/03/2007	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI390190	M	United States	2007-Aug-01	A/Wisconsin/03/2007	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI390193	NS	United States	2007-Aug-01	A/Wisconsin/03/2007	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI381903	NP	United States	2009-Aug-01	A/Wisconsin/15/2009	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI381902	M	United States	2009-Aug-01	A/Wisconsin/15/2009	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI381904	NS	United States	2009-Aug-01	A/Wisconsin/15/2009	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI367117	PB2	United States	2005-Jan-01	***A/Wisconsin/67/2005***	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI367116	PB1	United States	2005-Jan-01	***A/Wisconsin/67/2005***	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI367115	PA	United States	2005-Jan-01	***A/Wisconsin/67/2005***	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI367113	NP	United States	2005-Jan-01	***A/Wisconsin/67/2005***	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI367111	M	United States	2005-Jan-01	***A/Wisconsin/67/2005***	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI367114	NS	United States	2005-Jan-01	***A/Wisconsin/67/2005***	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI504828	PB2	United States	2003-Feb-13	A/Wyoming/03/2003	Iowa State Hygienic Laboratory	Centers for Disease Control and Prevention	
	EPI503943	PB1	United States	2003-Feb-13	A/Wyoming/03/2003	Iowa State Hygienic Laboratory	Centers for Disease Control and Prevention	
	EPI503942	PA	United States	2003-Feb-13	A/Wyoming/03/2003	Iowa State Hygienic Laboratory	Centers for Disease Control and Prevention	
	EPI499391	NP	United States	2003-Feb-13	A/Wyoming/03/2003	Iowa State Hygienic Laboratory	Centers for Disease Control and Prevention	
	EPI499392	M	United States	2003-Feb-13	A/Wyoming/03/2003	Iowa State Hygienic Laboratory	Centers for Disease Control and Prevention	
	EPI499393	NS	United States	2003-Feb-13	A/Wyoming/03/2003	Iowa State Hygienic Laboratory	Centers for Disease Control and Prevention	

(Table S4.2 cont.)

Influenza (sub)type	Segment ID	Segment	Country	Collection date (year-month-day)	Reference virus	Originating Lab	Submitting Lab	Authors
B	EPI366555	PB2	Bangladesh	2007-Jan-01	B/Bangladesh/3333/2007	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI366554	PB1	Bangladesh	2007-Jan-01	B/Bangladesh/3333/2007	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI366553	PA	Bangladesh	2007-Jan-01	B/Bangladesh/3333/2007	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI366551	NP	Bangladesh	2007-Jan-01	B/Bangladesh/3333/2007	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI366550	M	Bangladesh	2007-Jan-01	B/Bangladesh/3333/2007	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI366552	NS	Bangladesh	2007-Jan-01	B/Bangladesh/3333/2007	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI294715	NS	Bolivia, Plurinationial State of	2010-Jan-01	B/Bolivia/1526/2010	-	Centers for Disease Control and Prevention	-
	EPI370453	PB2	Australia	2008-Jul-13	B/Brisbane/33/2008	Queensland Health Scientific Services	WHO Collaborating Centre for Reference and Research on Influenza	-
	EPI370452	PB1	Australia	2008-Jul-13	B/Brisbane/33/2008	Queensland Health Scientific Services	WHO Collaborating Centre for Reference and Research on Influenza	-
	EPI370451	PA	Australia	2008-Jul-13	B/Brisbane/33/2008	Queensland Health Scientific Services	WHO Collaborating Centre for Reference and Research on Influenza	-
	EPI370450	NP	Australia	2008-Jul-13	B/Brisbane/33/2008	Queensland Health Scientific Services	WHO Collaborating Centre for Reference and Research on Influenza	-
	EPI370449	M	Australia	2008-Jul-13	B/Brisbane/33/2008	Queensland Health Scientific Services	WHO Collaborating Centre for Reference and Research on Influenza	-
	EPI370448	NS	Australia	2008-Jul-13	B/Brisbane/33/2008	Queensland Health Scientific Services	WHO Collaborating Centre for Reference and Research on Influenza	-
	EPI556423	PB2	Australia	2008-Aug-04	***B/Brisbane/60/2008***	Queensland Health Scientific Services	WHO Collaborating Centre for Reference and Research on Influenza	-
	EPI556424	PB1	Australia	2008-Aug-04	***B/Brisbane/60/2008***	Queensland Health Scientific Services	WHO Collaborating Centre for Reference and Research on Influenza	-
	EPI556425	PA	Australia	2008-Aug-04	***B/Brisbane/60/2008***	Queensland Health Scientific Services	WHO Collaborating Centre for Reference and Research on Influenza	-
	EPI309756	NP	Australia	2008-Aug-04	***B/Brisbane/60/2008***	Queensland Health Scientific Services	WHO Collaborating Centre for Reference and Research on Influenza	-
	EPI217339	M	Australia	2008-Aug-04	***B/Brisbane/60/2008***	Queensland Health Scientific Services	WHO Collaborating Centre for Reference and Research on Influenza	-
	EPI309757	NS	Australia	2008-Aug-04	***B/Brisbane/60/2008***	Queensland Health Scientific Services	WHO Collaborating Centre for Reference and Research on Influenza	-
	EPI348436	NS	Cambodia	2011-Oct-10	B/Cambodia/1412/2011	National Public Health Laboratory	Centers for Disease Control and Prevention	-
	EPI366571	PB2	United Kingdom	2008-Jan-01	B/England/145/2008	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI366570	PB1	United Kingdom	2008-Jan-01	B/England/145/2008	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI366569	PA	United Kingdom	2008-Jan-01	B/England/145/2008	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI366567	NP	United Kingdom	2008-Jan-01	B/England/145/2008	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI366565	M	United Kingdom	2008-Jan-01	B/England/145/2008	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI366568	NS	United Kingdom	2008-Jan-01	B/England/145/2008	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI406981	NS	Estonia	2011-Mar-14	B/Estonia/55669/2011	National Institute for Medical Research	Centers for Disease Control and Prevention	-
	EPI294741	NS	United States	2010-Oct-18	B/Florida/04/2010	Florida Department of Health-Jacksonville	Centers for Disease Control and Prevention	-
	EPI159985	PB2	United States	2006-Jan-01	***B/Florida/04/2006***	-	Other Database Import	-
	EPI159984	PB1	United States	2006-Jan-01	***B/Florida/04/2006***	-	Other Database Import	-
	EPI159983	PA	United States	2006-Jan-01	***B/Florida/04/2006***	-	Other Database Import	-
	EPI159981	NP	United States	2006-Jan-01	***B/Florida/04/2006***	-	Other Database Import	-
	EPI159979	M	United States	2006-Jan-01	***B/Florida/04/2006***	-	Other Database Import	-
	EPI159982	NS	United States	2006-Jan-01	***B/Florida/04/2006***	-	Other Database Import	-
	EPI366587	PB2	China	2008-Jan-01	B/Fujian-Gulou/1272/2008	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI366586	PB1	China	2008-Jan-01	B/Fujian-Gulou/1272/2008	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI366585	PA	China	2008-Jan-01	B/Fujian-Gulou/1272/2008	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI366583	NP	China	2008-Jan-01	B/Fujian-Gulou/1272/2008	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI366581	M	China	2008-Jan-01	B/Fujian-Gulou/1272/2008	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI366584	NS	China	2008-Jan-01	B/Fujian-Gulou/1272/2008	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI51248	PB2	United States	2004-Jun-21	B/Hawaii/13/2004	-	Other Database Import	-
	EPI51246	PB1	United States	2004-Jun-21	B/Hawaii/13/2004	-	Other Database Import	-
	EPI51244	PA	United States	2004-Jun-21	B/Hawaii/13/2004	-	Other Database Import	-
	EPI51239	NP	United States	2004-Jun-21	B/Hawaii/13/2004	-	Other Database Import	-
	EPI51233	M	United States	2004-Jun-21	B/Hawaii/13/2004	-	Other Database Import	-
	EPI51241	NS	United States	2004-Jun-21	B/Hawaii/13/2004	-	Other Database Import	-
	EPI301200	NS	Honduras	2010-Oct-11	B/Honduras/6927/2010	Laboratorio Nacional de Virologia	Centers for Disease Control and Prevention	-
	EPI232752	NS	China	2009-Feb-23	B/Jiangsu-Gulou/125/2009	WHO Chinese National Influenza Center	Centers for Disease Control and Prevention	-
	CY033851*	PB2	China	2003	B/Jiangsu/10/2003	-	-	Spiro,D.; Halpin,R.; Boyne,A.; Bera,J.; Ghedin,E.; *et al.*
	CY033850*	PB1	China	2003	B/Jiangsu/10/2003	-	-	Spiro,D.; Halpin,R.; Boyne,A.; Bera,J.; Ghedin,E.; *et al.*
	CY033849*	PA	China	2003	B/Jiangsu/10/2003	-	-	Spiro,D.; Halpin,R.; Boyne,A.; Bera,J.; Ghedin,E.; *et al.*
	CY033847*	NP	China	2003	B/Jiangsu/10/2003	-	-	Spiro,D.; Halpin,R.; Boyne,A.; Bera,J.; Ghedin,E.; *et al.*
	CY033845*	M	China	2003	B/Jiangsu/10/2003	-	-	Spiro,D.; Halpin,R.; Boyne,A.; Bera,J.; Ghedin,E.; *et al.*
	CY033848*	NS	China	2003	B/Jiangsu/10/2003	-	-	Spiro,D.; Halpin,R.; Boyne,A.; Bera,J.; Ghedin,E.; *et al.*
	EPI159937	PB2	China	2003-Jan-01	B/Jilin/20/2003	-	Other Database Import	-
	EPI159936	PB1	China	2003-Jan-01	B/Jilin/20/2003	-	Other Database Import	-
	EPI159935	PA	China	2003-Jan-01	B/Jilin/20/2003	-	Other Database Import	-
	EPI159933	NP	China	2003-Jan-01	B/Jilin/20/2003	-	Other Database Import	-
	EPI159931	M	China	2003-Jan-01	B/Jilin/20/2003	-	Other Database Import	-
	EPI159934	NS	China	2003-Jan-01	B/Jilin/20/2003	-	Other Database Import	-

(Table S4.2 cont.)

Influenza (sub)type	Segment ID	Segment	Country	Collection date (year-month-day)	Reference virus	Originating Lab	Submitting Lab	Authors
B	EPI294799	NS	Lao, People's Democratic Republic	2010-Sep-14	B/Laos/833/2010	National Center for Laboratory and Epidemiology	Centers for Disease Control and Prevention	-
	EPI175762	PB2	Malaysia	2004-Jan-01	***B/Malaysia/2506/2004***	-	Other Database Import	-
	EPI175761	PB1	Malaysia	2004-Jan-01	***B/Malaysia/2506/2004***	-	Other Database Import	-
	EPI175760	PA	Malaysia	2004-Jan-01	***B/Malaysia/2506/2004***	-	Other Database Import	-
	EPI175758	NP	Malaysia	2004-Jan-01	***B/Malaysia/2506/2004***	-	Other Database Import	-
	EPI175756	M	Malaysia	2004-Jan-01	***B/Malaysia/2506/2004***	-	Other Database Import	-
	EPI175759	NS	Malaysia	2004-Jan-01	***B/Malaysia/2506/2004***	-	Other Database Import	-
	EPI397596	PB2	United States	2012-Mar-13	***B/Massachusetts/02/2012***	Massachusetts Department of Public Health	Centers for Disease Control and Prevention	-
	EPI395218	PB1	United States	2012-Mar-13	***B/Massachusetts/02/2012***	Massachusetts Department of Public Health	Centers for Disease Control and Prevention	-
	EPI395217	PA	United States	2012-Mar-13	***B/Massachusetts/02/2012***	Massachusetts Department of Public Health	Centers for Disease Control and Prevention	-
	EPI395214	NP	United States	2012-Mar-13	***B/Massachusetts/02/2012***	Massachusetts Department of Public Health	Centers for Disease Control and Prevention	-
	EPI395216	M	United States	2012-Mar-13	***B/Massachusetts/02/2012***	Massachusetts Department of Public Health	Centers for Disease Control and Prevention	-
	EPI395215	NS	United States	2012-Mar-13	***B/Massachusetts/02/2012***	Massachusetts Department of Public Health	Centers for Disease Control and Prevention	-
	EPI406959	NS	United States	2012-Oct-28	B/Montana/06/2012	Montana Laboratory Services Bureau	Centers for Disease Control and Prevention	-
	EPI407350	NS	United States	2012-Nov-26	B/New Mexico/04/2012	New Mexico Department of Health	Centers for Disease Control and Prevention	-
	EPI368750	NS	Russian Federation	2012-Feb-14	B/Novosibirsk/01/2012	Russian Academy of Medical Sciences	Centers for Disease Control and Prevention	-
	EPI346476	PB2	United States	2005-Feb-23	B/Ohio/01/2005	Cincinnati Children's Hospital	Centers for Disease Control and Prevention	-
	EPI346475	PB1	United States	2005-Feb-23	B/Ohio/01/2005	Cincinnati Children's Hospital	Centers for Disease Control and Prevention	-
	EPI346477	PA	United States	2005-Feb-23	B/Ohio/01/2005	Cincinnati Children's Hospital	Centers for Disease Control and Prevention	-
	EPI346469	NP	United States	2005-Feb-23	B/Ohio/01/2005	Cincinnati Children's Hospital	Centers for Disease Control and Prevention	-
	EPI346471	M	United States	2005-Feb-23	B/Ohio/01/2005	Cincinnati Children's Hospital	Centers for Disease Control and Prevention	-
	EPI346520	NS	United States	2005-Feb-23	B/Ohio/01/2005	Cincinnati Children's Hospital	Centers for Disease Control and Prevention	-
	AJ781208*	PB2	Norway	2004	B/Oslo/71/2004	-	National Institute for Medical Research	Wright,H.G., Gregory,V., Lin,Y., Bennett,M. and Hay,A.J.
	AJ781187*	PB1	Norway	2004	B/Oslo/71/2004	-	National Institute for Medical Research	Wright,H.G., Gregory,V., Lin,Y., Bennett,M. and Hay,A.J.
	AJ716222*	PA	Norway	2004	B/Oslo/71/2004	-	National Institute for Medical Research	Wright,H.G., Gregory,V., Lin,Y., Bennett,M. and Hay,A.J.
	AJ784079*	NP	Norway	2004	B/Oslo/71/2004	-	National Institute for Medical Research	Wright,H.G., Gregory,V., Lin,Y., Bennett,M. and Hay,A.J.
	AJ783391*	M	Norway	2004	B/Oslo/71/2004	-	National Institute for Medical Research	Wright,H.G., Gregory,V., Lin,Y., Bennett,M. and Hay,A.J.
	AJ781288*	NS	Norway	2004	B/Oslo/71/2004	-	National Institute for Medical Research	Wright,H.G., Gregory,V., Lin,Y., Bennett,M. and Hay,A.J.
	EPI13407	PB2	China	2002-Jun-12	***B/Shanghai/361/2002***	-	Other Database Import	-
	EPI13365	PB1	China	2002-Jun-12	***B/Shanghai/361/2002***	-	Other Database Import	-
	EPI13647	NP	China	2002-Jun-12	***B/Shanghai/361/2002***	-	Other Database Import	-
	EPI13553	M	China	2002-Jun-12	***B/Shanghai/361/2002***	-	Other Database Import	-
	EPI13467	NS	China	2002-Jun-12	***B/Shanghai/361/2002***	-	Other Database Import	-
	EPI340832	NS	Sweden	2011-Feb-28	B/Stockholm/12/2011	National Institute for Medical Research	Centers for Disease Control and Prevention	-
	EPI241184	NS	Uganda	2009-Oct-21	B/Uganda/0514/2009	Uganda Virus Research Institute (UVRI), National Influenza Center	Centers for Disease Control and Prevention	-
	EPI502818	PB2	Australia	2006-Jun-06	B/Victoria/304/2006	-	Other Database Import	Wentworth,D.E.; Halpin,R.A.; Lin,X.; Zhou,B.; Bera,J.; *et al.*
	EPI502817	PB1	Australia	2006-Jun-06	B/Victoria/304/2006	-	Other Database Import	Wentworth,D.E.; Halpin,R.A.; Lin,X.; Zhou,B.; Bera,J.; *et al.*
	EPI502816	PA	Australia	2006-Jun-06	B/Victoria/304/2006	-	Other Database Import	Wentworth,D.E.; Halpin,R.A.; Lin,X.; Zhou,B.; Bera,J.; *et al.*
	EPI502814	NP	Australia	2006-Jun-06	B/Victoria/304/2006	-	Other Database Import	Wentworth,D.E.; Halpin,R.A.; Lin,X.; Zhou,B.; Bera,J.; *et al.*
	EPI502812	M	Australia	2006-Jun-06	B/Victoria/304/2006	-	Other Database Import	Wentworth,D.E.; Halpin,R.A.; Lin,X.; Zhou,B.; Bera,J.; *et al.*
	EPI502815	NS	Australia	2006-Jun-06	B/Victoria/304/2006	-	Other Database Import	Wentworth,D.E.; Halpin,R.A.; Lin,X.; Zhou,B.; Bera,J.; *et al.*
	EPI483011	PB2	Australia	2005-Oct-16	B/Victoria/530/2005	-	Other Database Import	Wentworth,D.E.; Halpin,R.A.; Lin,X.; Zhou,B.; Bera,J.; *et al.*
	EPI483010	PB1	Australia	2005-Oct-16	B/Victoria/530/2005	-	Other Database Import	Wentworth,D.E.; Halpin,R.A.; Lin,X.; Zhou,B.; Bera,J.; *et al.*
	EPI483009	PA	Australia	2005-Oct-16	B/Victoria/530/2005	-	Other Database Import	Wentworth,D.E.; Halpin,R.A.; Lin,X.; Zhou,B.; Bera,J.; *et al.*
	EPI483007	NP	Australia	2005-Oct-16	B/Victoria/530/2005	-	Other Database Import	Wentworth,D.E.; Halpin,R.A.; Lin,X.; Zhou,B.; Bera,J.; *et al.*
	EPI483005	M	Australia	2005-Oct-16	B/Victoria/530/2005	-	Other Database Import	Wentworth,D.E.; Halpin,R.A.; Lin,X.; Zhou,B.; Bera,J.; *et al.*
	EPI483008	NS	Australia	2005-Oct-16	B/Victoria/530/2005	-	Other Database Import	Wentworth,D.E.; Halpin,R.A.; Lin,X.; Zhou,B.; Bera,J.; *et al.*
	EPI366487	PB2	United States	2010-Jan-01	***B/Wisconsin/01/2010***	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI366486	PB1	United States	2010-Jan-01	***B/Wisconsin/01/2010***	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI366485	PA	United States	2010-Jan-01	***B/Wisconsin/01/2010***	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI366483	NP	United States	2010-Jan-01	***B/Wisconsin/01/2010***	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI366481	M	United States	2010-Jan-01	***B/Wisconsin/01/2010***	-	Other Database Import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; *et al.*
	EPI271598	NS	United States	2010-Feb-20	***B/Wisconsin/01/2010***	Wisconsin State Laboratory of Hygiene	Centers for Disease Control and Prevention	-
A(H1N1)pdm09	EPI176616	PB2	United States	2009-Apr-09	***A/California/07/2009***	Naval Health Research Center	Centers for Disease Control and Prevention	-
	EPI176615	PB1	United States	2009-Apr-09	***A/California/07/2009***	Naval Health Research Center	Centers for Disease Control and Prevention	-
	EPI176617	PA	United States	2009-Apr-09	***A/California/07/2009***	Naval Health Research Center	Centers for Disease Control and Prevention	-
	EPI184298	NP	United States	2009-Apr-09	***A/California/07/2009***	Naval Health Research Center	Centers for Disease Control and Prevention	-
	EPI176619	M	United States	2009-Apr-09	***A/California/07/2009***	Naval Health Research Center	Centers for Disease Control and Prevention	-
	EPI176618	NS	United States	2009-Apr-09	***A/California/07/2009***	Naval Health Research Center	Centers for Disease Control and Prevention	-
	EPI190211	PB2	Denmark	2009-Jun-09	A/Denmark/528/2009	-	Other Database Import	-
	EPI190212	PB1	Denmark	2009-Jun-09	A/Denmark/528/2009	-	Other Database Import	-

(Table S4.2 cont.)

Influenza (sub)type	Segment ID	Segment	Country	Collection date (year-month-day)	Reference virus	Originating Lab	Submitting Lab	Authors
A(H1N1)pdm09	EPI190213	PA	Denmark	2009-Jun-09	A/Denmark/528/2009	-	Other Database Import	-
	EPI190215	NP	Denmark	2009-Jun-09	A/Denmark/528/2009	-	Other Database Import	-
	EPI190217	M	Denmark	2009-Jun-09	A/Denmark/528/2009	-	Other Database Import	-
	EPI190218	NS	Denmark	2009-Jun-09	A/Denmark/528/2009	-	Other Database Import	-
	EPI189163	M	Singapore	2009-May-30	A/Singapore/57/2009	Singapore General Hospital	Centers for Disease Control and Prevention	-
	EPI177279	M	New Zealand	2009-Apr-25	A/Auckland/03/2009	Auckland Hospital	WHO Collaborating Centre for Reference and Research on Influenza	-
	EPI239629	M	Germany	2009-Jan-01	A/Bayern/69/2009	-	Centers for Disease Control and Prevention	-
	EPI215955	PB2	Ukraine	2009-Oct-27	A/Lviv/N6/2009	Ministry of Health of Ukraine	National Institute for Medical Research	-
	EPI215952	NP	Ukraine	2009-Oct-27	A/Lviv/N6/2009	Ministry of Health of Ukraine	National Institute for Medical Research	-
	EPI215954	M	Ukraine	2009-Oct-27	A/Lviv/N6/2009	Ministry of Health of Ukraine	National Institute for Medical Research	-
	EPI215953	NS	Ukraine	2009-Oct-27	A/Lviv/N6/2009	Ministry of Health of Ukraine	National Institute for Medical Research	-
	EPI233200	M	United States	2009-Oct-16	A/North Carolina/49/2009	North Carolina State Laboratory of Public Health	Centers for Disease Control and Prevention	-
	EPI239641	M	Vietnam	2009-Dec-01	A/Vietnam/2043/2009	National Institute of Hygiene and Epidemiology	Centers for Disease Control and Prevention	-
	EPI280342	M	New Zealand	2010-Jul-12	A/Christchurch/16/2010	WHO Collaborating Centre for Reference and Research on Influenza	Centers for Disease Control and Prevention	-
	EPI294672	PB2	United Kingdom	2010-Nov-01	A/England/4880374/2010	Microbiology Services Colindale, Public Health England	Microbiology Services Colindale, Public Health England	Galiano,M.
	EPI294671	PA	United Kingdom	2010-Nov-01	A/England/4880374/2010	Microbiology Services Colindale, Public Health England	Microbiology Services Colindale, Public Health England	Galiano,M.
	EPI294668	NP	United Kingdom	2010-Nov-01	A/England/4880374/2010	Microbiology Services Colindale, Public Health England	Microbiology Services Colindale, Public Health England	Galiano,M.
	EPI294670	M	United Kingdom	2010-Nov-01	A/England/4880374/2010	Microbiology Services Colindale, Public Health England	Microbiology Services Colindale, Public Health England	Galiano,M.
	EPI294669	NS	United Kingdom	2010-Nov-01	A/England/4880374/2010	Microbiology Services Colindale, Public Health England	Microbiology Services Colindale, Public Health England	-
	EPI342431	PB2	United States	2010-Nov-01	A/Kentucky/09/2010	Kentucky Division of Laboratory Services	Centers for Disease Control and Prevention	-
	EPI342432	PB1	United States	2010-Nov-01	A/Kentucky/09/2010	Kentucky Division of Laboratory Services	Centers for Disease Control and Prevention	-
	EPI342430	PA	United States	2010-Nov-01	A/Kentucky/09/2010	Kentucky Division of Laboratory Services	Centers for Disease Control and Prevention	-
	EPI342427	NP	United States	2010-Nov-01	A/Kentucky/09/2010	Kentucky Division of Laboratory Services	Centers for Disease Control and Prevention	-
	EPI342429	M	United States	2010-Nov-01	A/Kentucky/09/2010	Kentucky Division of Laboratory Services	Centers for Disease Control and Prevention	-
	EPI342428	NS	United States	2010-Nov-01	A/Kentucky/09/2010	Kentucky Division of Laboratory Services	Centers for Disease Control and Prevention	-
	EPI301382	M	Sweden	2010-Dec-02	A/Stockholm/12/2010	Swedish Institute for Infectious Disease Control	Swedish Institute for Infectious Disease Control	-
	EPI301781	M	Sweden	2010-Dec-12	A/Stockholm/14/2010	Swedish Institute for Infectious Disease Control	Swedish Institute for Infectious Disease Control	-
	EPI252104	PB2	United States	2010-Feb-08	A/Wyoming/01/2010	Wyoming Public Health Laboratory	Centers for Disease Control and Prevention	-
	EPI252105	PB1	United States	2010-Feb-08	A/Wyoming/01/2010	Wyoming Public Health Laboratory	Centers for Disease Control and Prevention	-
	EPI252103	PA	United States	2010-Feb-08	A/Wyoming/01/2010	Wyoming Public Health Laboratory	Centers for Disease Control and Prevention	-
	EPI252101	NP	United States	2010-Feb-08	A/Wyoming/01/2010	Wyoming Public Health Laboratory	Centers for Disease Control and Prevention	-
	EPI247504	M	United States	2010-Feb-08	A/Wyoming/01/2010	Wyoming Public Health Laboratory	Centers for Disease Control and Prevention	-
	EPI252102	NS	United States	2010-Feb-08	A/Wyoming/01/2010	Wyoming Public Health Laboratory	Centers for Disease Control and Prevention	-
	EPI348183	M	United States	2011-Dec-07	A/Florida/35/2011	Florida Department of Health-Tampa	Centers for Disease Control and Prevention	-
	EPI346511	M	Thailand	2011-Sep-12	A/Song Khla/270/2011	WHO National Influenza Centre, National Institute of Medical Research	Centers for Disease Control and Prevention	-
	EPI316432	PB2	Russian Federation	2011-Mar-14	A/St. Petersburg/100/2011	Russian Academy of Medical Sciences	Centers for Disease Control and Prevention	-
	EPI316433	PB1	Russian Federation	2011-Mar-14	A/St. Petersburg/100/2011	Russian Academy of Medical Sciences	Centers for Disease Control and Prevention	-
	EPI316431	PA	Russian Federation	2011-Mar-14	A/St. Petersburg/100/2011	Russian Academy of Medical Sciences	Centers for Disease Control and Prevention	-
	EPI316428	NP	Russian Federation	2011-Mar-14	A/St. Petersburg/100/2011	Russian Academy of Medical Sciences	Centers for Disease Control and Prevention	-
	EPI316430	M	Russian Federation	2011-Mar-14	A/St. Petersburg/100/2011	Russian Academy of Medical Sciences	Centers for Disease Control and Prevention	-
	EPI316429	NS	Russian Federation	2011-Mar-14	A/St. Petersburg/100/2011	Russian Academy of Medical Sciences	Centers for Disease Control and Prevention	-
	EPI335838	M	Chile	2011-Aug-10	A/Valparaiso/17275/2011	Instituto de Salud Publica de Chile	Centers for Disease Control and Prevention	-
	EPI500464	M	Senegal	2012-Dec-09	A/Dakar/20/2012	Institut Pasteur de Dakar	National Institute for Medical Research	-
	EPI382426	M	Hong Kong (SAR)	2012-May-21	A/Hong Kong/5659/2012	Public Health Laboratory Services Branch, Centre for Health Protection	Public Health Laboratory Services Branch, Centre for Health Protection	Mak,G.C.;Lo,J.Y.C.
	EPI500493	M	France	2012-Dec-28	A/Lyon/02/2013	CRR virus Influenza region Sud	National Institute for Medical Research	-
	EPI500516	M	Norway	2012-Dec-17	A/Norway/2552/2012	WHO National Influenza Centre	National Institute for Medical Research	-
	EPI347566	M	Sweden	2011-Nov-22	A/Stockholm/35/2011	-	Swedish Institute for Infectious Disease Control	-
	EPI406024	M	United States	2012-Oct-31	A/Tennessee/09/2012	Tennessee Department of Health Laboratory-Nashville	Centers for Disease Control and Prevention	-

Virtually all sequences were retrieved from GISAID EpiFlu™ database. The sequences exceptionally retrieved from NCBI Influenza Virus Resource database are marked with an asterisk (*) after the accession number (2nd column). Reference viruses included in the Northern Hemisphere´s seasonal influenza vaccines are highlighted in bold and italic. The dash (-) represents no information.

CHAPTER 6

UNCOVERING THE MOLECULAR FOOTPRINTS OF SELECTIVE PRESSURE IN INFLUENZA A AND B VIRUS NEURAMINIDASE GENE

Results partially presented at:

Correia V, Abecasis AB, Rebelo-de-Andrade H, 2016. Uncovering the role of positive selection in the evolutionary pathways to influenza virus resistance or reduced inhibition by neuraminidase inhibitors. Poster presentation at the Options IX for the Control of Influenza, Chicago, USA, 26th August.

Correia V, Abecasis AB, Rebelo-de-Andrade H, 2014. Selective pressure acting on influenza neuraminidase gene: a complementary approach for studying the emergence and evolution of antiviral drug resistance and reduced susceptibility. Oral presentation at the International Meeting on Emerging Diseases and Surveillance, Vienna, Austria, 3rd November.

Correia V, Abecasis AB, Rebelo-de-Andrade H, 2014. Large-scale differential selection analysis on influenza A and B neuraminidase gene: a new approach for studying antiviral drug resistance and reduced susceptibility. Oral presentation at the III International Conference on Antimicrobial Research, Madrid, Spain, 3rd October.

I carried out all activities, methodologies and data analysis underlying the results presented in this chapter. The phylogenetic trees and both site-specific and differential selective pressure analysis of NA sequence (sub-)datasets with more than 3000 sequences were exceptionally constructed/executed at more powerful computer systems available at the laboratory of Ana Abecasis at the Instituto de Higiene e Medicina Tropical, Universidade NOVA de Lisboa. Ana Abecasis also provided key guidance and support during the entire work.

6 UNCOVERING THE MOLECULAR FOOTPRINTS OF SELECTIVE PRESSURE IN INFLUENZA A AND B VIRUS NEURAMINIDASE GENE

"One general law, leading to the advancement of all organic beings, namely, multiply, vary, let the strongest live and the weakest die."

Charles Darwin, On The Origin of Species, 1859

This chapter presents and discusses the results of the large-scale study conducted to analyse the selective pressure (SP) acting on the influenza neuraminidase (NA) of worldwide circulating viruses from all human influenza subtypes and lineages (former seasonal A(H1N1), A(H3N2), B/Victoria and B/Yamagata-lineage, and A(H1N1)pdm09) (3rd general objective; see section 2.2, Study Description). A higher focus was given to the SP acting on the codon sites (herein designated as sites) associated with NA inhibitor (NAI) resistance or (highly) reduced inhibition ((H)RI) *in vitro*, and on further sites contacting directly or indirectly with the drug (NA active site).

An overview of the main activities carried out during this study is presented below for a better understanding of the underlying work.

BOX 6.1 - MAIN ACTIVITIES

- Collection of all potentially complete NA coding sequences available at GISAID EpiFlu™ and NCBI Influenza Virus Resource databases, for each time period (temporal split) and influenza virus subtype or lineage. Addition of unpublished sequences of viruses circulating in Portugal.
- Five-step sequence treatment of temporal sub-datasets, followed by merging into total datasets.
- Inference of the maximum-likelihood phylogenetic tree from each total and temporal NA sequence (sub-)dataset, after determining the best-fit codon model for human influenza NA.
- Estimate the global and site-specific (SLAC and FEL analysis) ratios between non-synonymous (dN) and synonymous (dS) substitution rates (dN/dS ratios), for each total and temporal NA sequence (sub-)dataset.
- Identification and mapping of the positively selected sites onto the three-dimensional structure of the corresponding NA protein.
- Differential selection analysis to identify which sites within each influenza subtype or lineage NA evolved under different SP among the different contexts of NAI global use (temporal split).
- Determination of the frequency of amino acid substitutions conferring or enhancing resistance or (H)RI by NAIs *in vitro*.

6.1 RESULTS

The results are organized in 5 main sub-sections as follows: (1) study sample; (2) overall analysis of selective pressure; (3) site-by-site analysis of selective pressure; (4) differential analysis of site-by-site selective pressures among different contexts of neuraminidase inhibitor drug use (temporal sub-datasets); (5) frequency of amino acid substitutions conferring or enhancing neuraminidase inhibitor resistance or (highly) reduced inhibition *in vitro* in sequence databases (complementary study).

6.1.1 Study Sample

Following sequence treatment (detailed in section 3.2.3.2, Material and Methods), temporal sub-datasets contained between 34 and 3122 NA gene sequences of influenza viruses circulating worldwide (Table 6.1). The number of sequences varied widely not only among human influenza virus subtypes or lineages but also time periods, as already evidenced in the original sequence sub-datasets (see Table 3.3, Material and Methods). Only the two later A(H3N2) NA sequence sub-datasets were comprised by a similar number of sequences. The first temporal sub-dataset (before 1999) contained a much smaller number of sequences for all virus subtypes and lineages (not applied to A(H1N1)pdm09 subtype) (Table 6.1). It included sequences from 1943 onwards for former seasonal A(H1N1) subtype (herein designated as seasonal A(H1N1)); from 1968 onwards for A(H3N2) subtype, matching the beginning of virus circulation; and from 1972 and 1973 onwards for, respectively, influenza B/Victoria (B/VIC) and B/Yamagata (B/YAM) lineages, covering the beginning of lineage differentiation. A(H3N2) temporal sub-datasets contained a higher number of sequences compared to seasonal A(H1N1) and both influenza B/VIC and B/YAM-lineage sub-datasets (Table 6.1). Seasonal A(H1N1) viruses ended to be completely replaced by the novel A(H1N1)pdm09 viruses, explaining why the latest sequences dated from 2010 (from 2009 onwards sub-dataset). The A(H1N1)pdm09 (pre-)pandemic[A] sub-dataset enclosed the highest number of sequences (3122 NA gene sequences).

Total NA sequence datasets were obtained by merging the treated temporal sub-datasets. An additional 99.99% redundancy threshold had to be applied to both A(H3N2) and

[A] Pre-pandemic (late March to 10th June 2009) or pandemic (11th June 2009 to 9th August 2010) period.

A(H1N1)pdm09 datasets to reduce the total number of sequences to below 4000 due to posterior software constraints (detailed in section 3.2.3.2, Material and Methods). A(H3N2) and A(H1N1)pdm09 total datasets were comprised by more than 3400 NA gene sequences, while the number of sequences within seasonal A(H1N1) and influenza B/VIC and B/YAM-lineage total datasets varied between approximately 1450 to 2000 sequences (Table 6.1).

Table 6.1 Characterization of the temporal sub-datasets of human influenza virus neuraminidase gene sequences after sequence treatment and of the resultant total sequence datasets, regarding number of sequences enclosed and time period covered.

Influenza virus subtype/lineage	TREATED TEMPORAL SUB-DATASETS								TOTAL DATASET	
	Before 1999		1999-2008		From 2009 onwards					
	N	T	N	T	N	T			N	T
Former seasonal A(H1N1)	94	1943 - 1998	1094	1999 - 2008	335	2009 - 2010			1523	1943 - 2010
A(H3N2)	665	1968 - 1998	1892	1999 - 2008	2027	2009 - 2013			3712 [b]	1968 - 2013
B/Victoria	34	1972 - 1998	384	1999 - 2008	1560	2009 - 2013			1978	1972 - 2013
B/Yamagata	83	1973 - 1998	414	1999 - 2008	944	2009 - 2013			1441	1973 - 2013
					(Pre-)pandemic period [a]		Post-pandemic period			
					N	T	N	T		
A(H1N1)pdm09	-	-	-	-	3122	2009 - 09/08/2010	1600	10/08/2010 - 2013	3428 [b]	2009 - 2013

N: Number of sequences; T: Time period

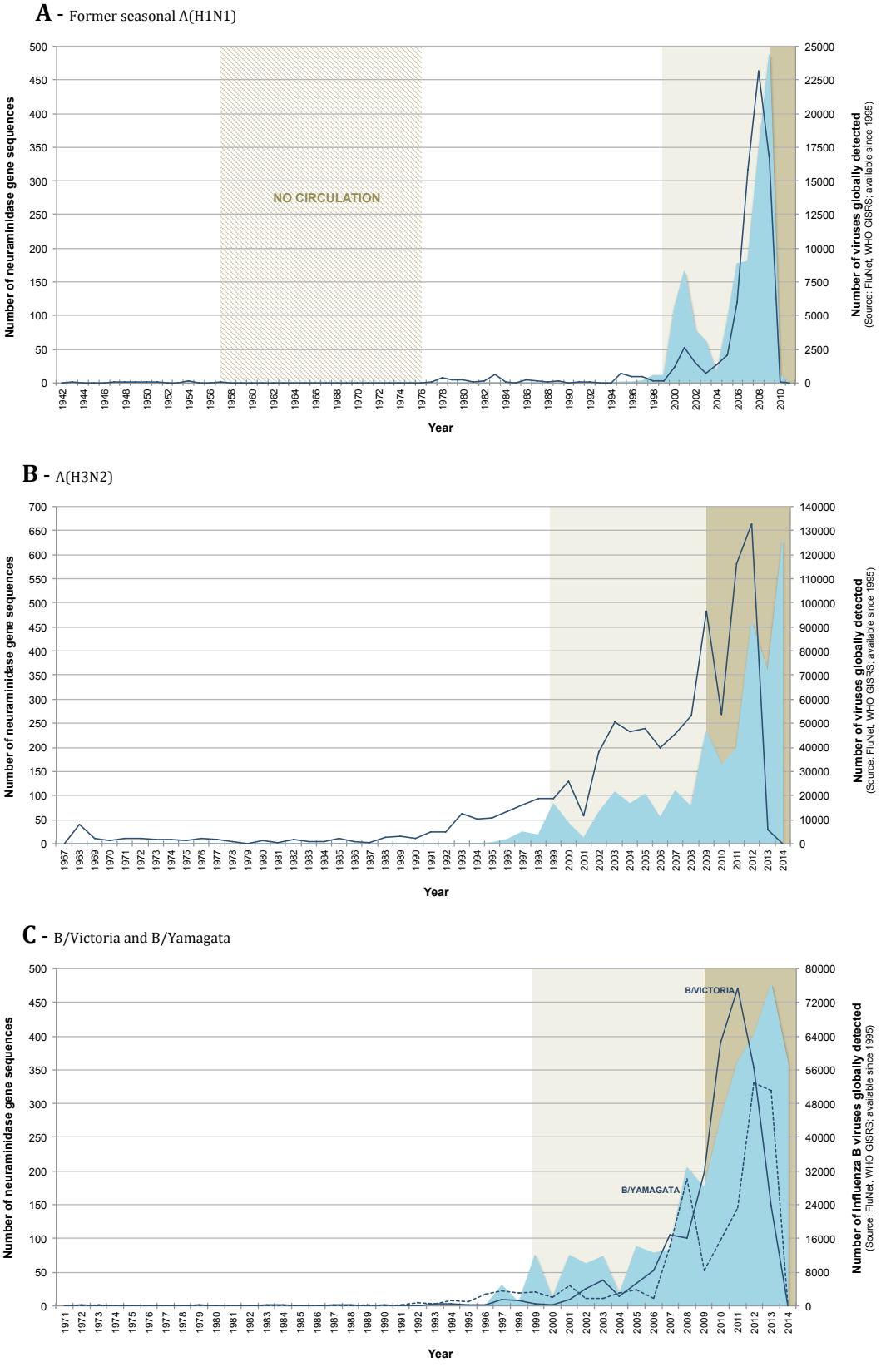
[a] Pre-pandemic or pandemic period; [b] Do not correspond to the sum of temporal sub-dataset sequences as a further 99.99% redundancy threshold had to be applied to reduce the number of sequences to below 4000 (software constraints in posterior selective pressure analysis).

The temporal split that defined the different temporal sub-datasets was based on differences on neuraminidase inhibitor drug use (detailed in section 2.1, Study Description).

6.1.1.1 Distribution Over Time

A(H3N2) NA sequence (sub-)datasets contained sequences from all years within the time period covered (Figure 6.1B). The same was verified for the two later seasonal A(H1N1) and influenza B/VIC and B/YAM temporal sub-datasets (1999-2008, from 2009 onwards), but not for the first one (before 1999) and in consequence for total dataset. Very few seasonal A(H1N1) NA sequences were from 1943 to 1956 (no circulation between 1957 and 1976), distributing sporadically across years, with the same occurring for B/VIC and B/YAM-lineage NA sequences from 1972 to 1992 and 1973 to 1991, respectively (Figure 6.1A,C). Sequences from most or all remaining years within the sub-dataset (before 1999) were present. Due to the smaller time period covered (≈2 to 5 years), the temporal

distribution of A(H1N1)pdm09 NA sequences was instead analysed by epidemiological week (Sunday to Saturday[1]) (Figure 6.1D). Sequences from all epidemiological weeks were present in A(H1N1)pdm09 (sub-)datasets.



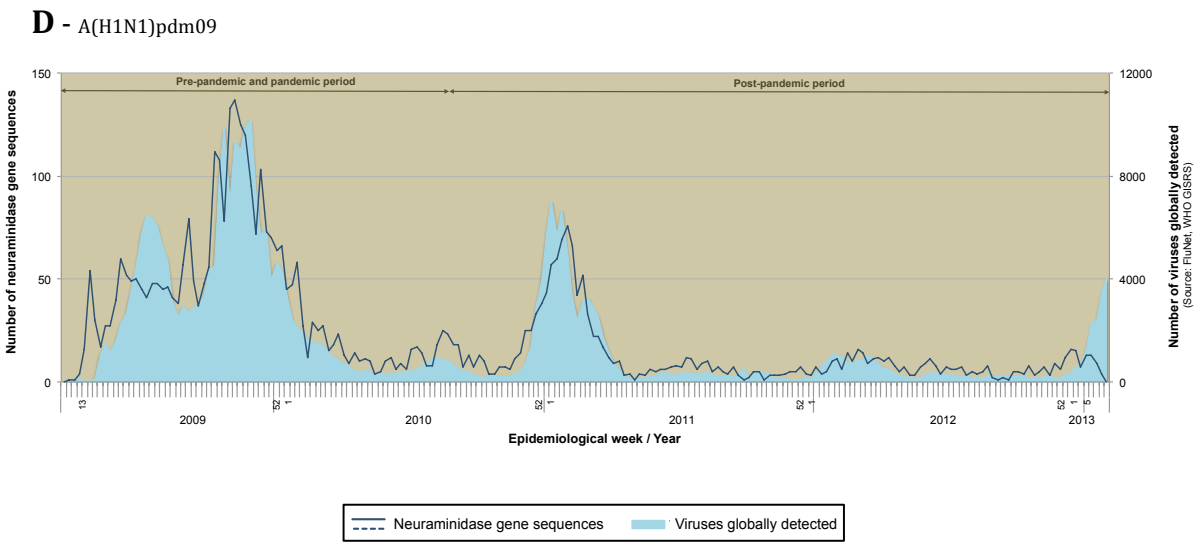


Figure 6.1 Temporal distribution of the neuraminidase gene sequences of former seasonal A(H1N1) **(A)**, A(H3N2) **(B)**, B/Victoria and B/Yamagata-lineage **(C)** and A(H1N1)pdm09 **(D)** sequence (sub-) datasets.

All sequences were analysed by year of virus collection except those belonging to A(H1N1)pdm09 subtype, for which the analysis was performed by epidemiological week (Sunday to Saturday[1]). Supplementary information on the number of virus globally detected is shown on the secondary y-axis for the time period for which this information was available (since 1995; Source: FluNet, World Health Organization (WHO) Global Influenza Surveillance and Response System (GISRS) (http://www.who.int/influenza/ gisrs_laboratory/flunet/en/)). **Panel A-C**: the different background colours indicate the time period covered the different temporal sub-datasets - white (before 1999); beige (1999-2008); golden brown (from 2009 onwards). **Panel D**: the time period covered by each temporal sub-dataset is indicated with arrows at the top of the plot.

The NA gene sequences of all human influenza virus subtype or lineage (sub-)datasets were not uniformly distributed over time (Figure 6.1A-D). This was, in part, related with the specific circulation dynamics of influenza viruses in human population, as evidenced by the overall overlapping variation in number of sequences and viruses globally detected (information available since 1995; Source: FluNet, World Health Organization (WHO) Global Influenza Surveillance and Response System [B]). Only for B/VIC lineage it seemed to exist since 2007 an one to two-year delay in the variation of both parameters (Figure 6.1C). The shift to right in the distribution of seasonal A(H1N1), A(H3N2) and influenza B/VIC and B/YAM-lineage NA sequences (Figure 6.1A-C), was already expected given the much smaller size of the earlier sub-dataset (before 1999). The fact that this earlier period was considerably longer than the two latter ones (26 to 56 years, compared to 10 and 2 to 5 years), made this shift even more evident.

[B] http://www.who.int/influenza/gisrs_laboratory/flunet/en/

6.1.1.2 Geographic Distribution (Continents and Influenza Transmission Zones)

All NA sequence (sub-)datasets contained sequences from the 5 worldwide continents (Americas, Europe, Africa, Asia and Oceania). The only exception was the earliest influenza B/VIC temporal sub-dataset (before 1999) that did not contained sequences from either Africa or Oceania (Figure 6.2). The distribution of the sequences across the different worldwide continents was not, however, uniform. Most (sub-)datasets were essentially comprised by sequences from the Americas, Asia and Europe, while others contained sequences from mainly the two former continents (seasonal A(H1N1) (sub-) datasets except 1999-2008, and influenza B/VIC and B/YAM before 1999 and 1999-2008 sub-datasets). Only very few of the sequences within each (sub-)dataset were from Africa (1% to 10%).

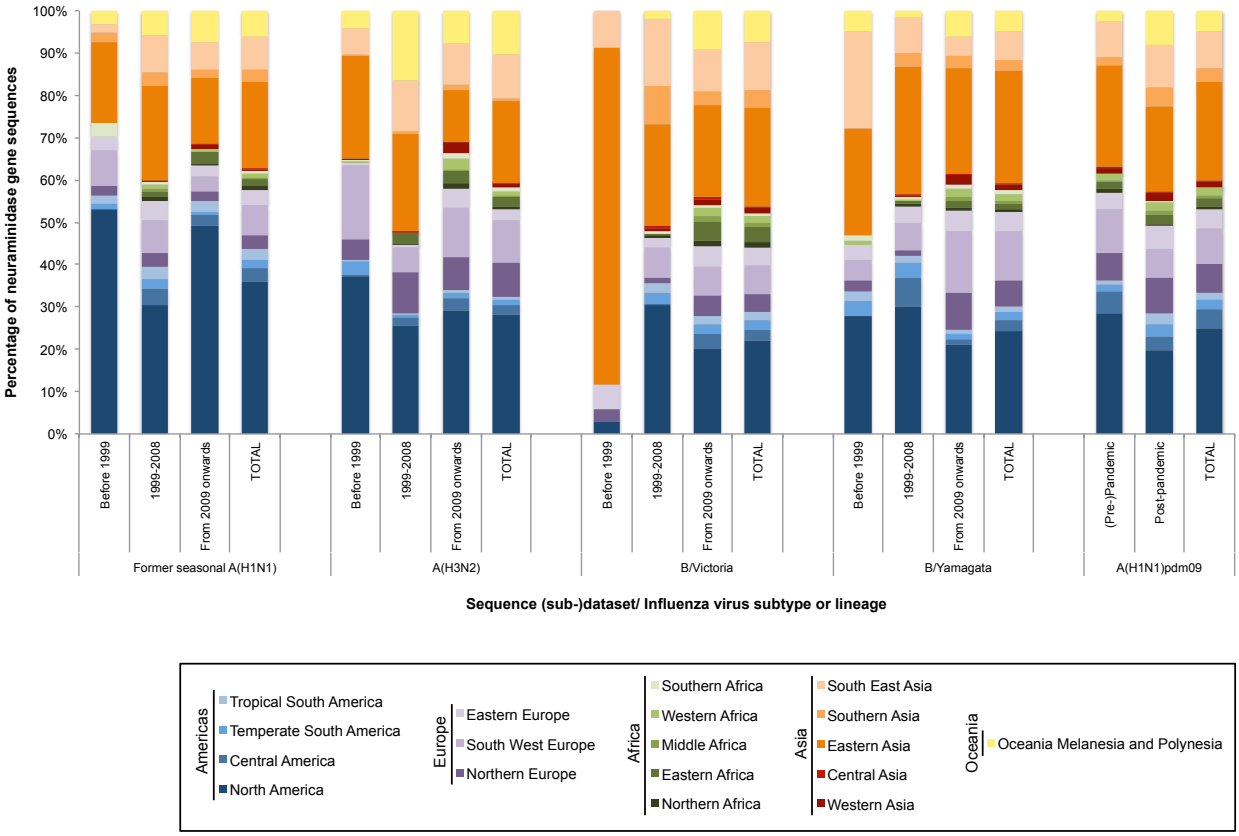


Figure 6.2 Geographic distribution of the neuraminidase gene sequences of former seasonal A(H1N1), A(H3N2), B/Victoria and B/Yamagata-lineage, and A(H1N1)pdm09 (sub-)datasets, by continent or influenza transmission zone.

The 18 influenza transmission zones established by the World Health Organization join geographically related countries or territories in larger areas with similar influenza transmission patterns [2].

A more detailed analysis showed that most (sub-)datasets contained sequences from all 18 influenza transmission zones established by WHO (Figure 6.2). An influenza transmission zone represents a geographical area with a similar influenza transmission pattern [2]. Most sequences within the (sub-)datasets belonged to the North America and Eastern Asia influenza transmission zones, with the third top position varying between South West Europe and South East Asia (Figure 6.2). As expected, given the lower number of sequences enclosed, the before 1999 sub-datasets were the less diversified, not comprising sequences from most influenza transmission zones in Africa.

6.1.1.3 Nucleotide Diversity

Nucleotide diversity[C] of NA sequence (sub-)datasets varied not only among human influenza virus subtypes or lineages but also over time (Figure 6.3). Similar degrees of polymorphism were exceptionally found for the influenza B/VIC and B/YAM total sequence datasets and also for the different temporal sub-datasets of B/YAM lineage.

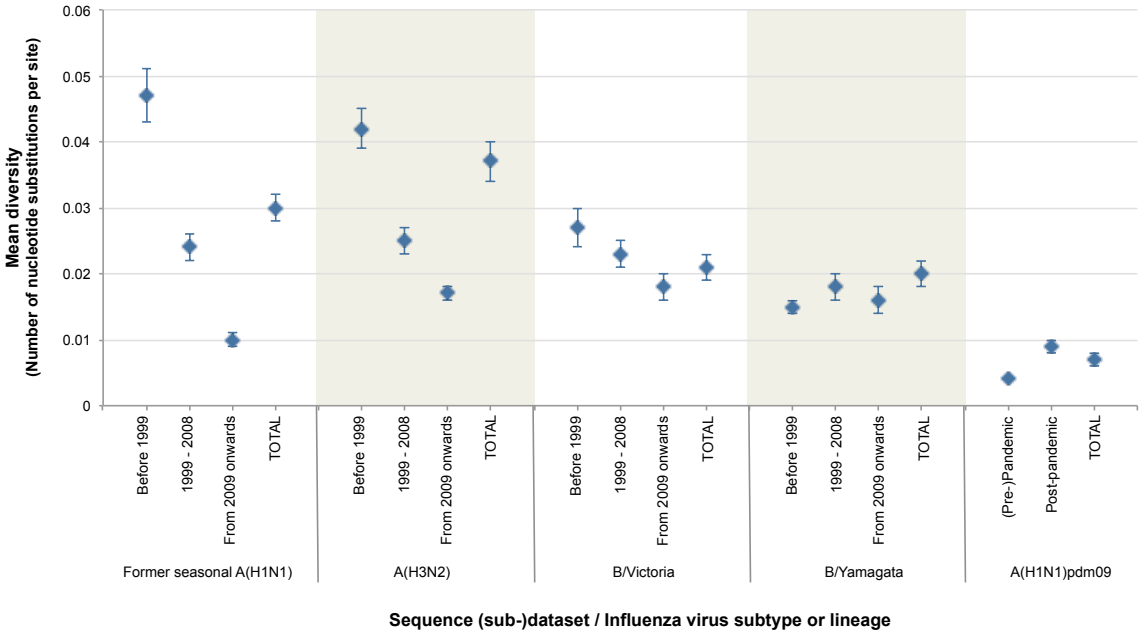


Figure 6.3 Mean nucleotide diversity of former seasonal A(H1N1), A(H3N2), B/Victoria and B/Yamagata-lineage, and A(H1N1)pdm09 neuraminidase sequence (sub-)datasets, with standard error estimates.

[C] Average number of nucleotide substitutions per site for all pairwise comparisons among the *n* sequences within the (sub-) dataset [3].

(Footnotes Figure 6.3)
Sequence diversity was estimated in MEGA5 software by Maximum Composite Likelihood method. Rate variation among sites was modelled with a gamma distribution (shape parameter = 1.327) and 500 bootstrap replications were used to estimate the associated standard error, represented in the figure by the error bars.

A(H3N2) sequence dataset was the most diverse of all human influenza subtype or lineage total datasets ($3.7x10^{-2}$ nucleotide substitutions per site), followed by seasonal A(H1N1) dataset ($3.0x10^{-2}$) and then by both influenza B/VIC and B/YAM datasets ($2.1x10^{-2}$ and $2.0x10^{-2}$, respectively). A(H1N1)pdm09 total sequence dataset was the less polymorphic, with a mean diversity of $0.7x10^{-2}$ nucleotide substitutions per site. The nucleotide sequence diversity of seasonal A(H1N1), A(H3N2) and, in a lesser extent, B/VIC-lineage temporal sub-datasets was increasingly lower, while the converse was observed for A(H1N1)pdm09 subtype. A(H1N1)pdm09 post-pandemic sub-dataset was approximately two times more diverse ($0.9x10^{-2}$) than the (pre-)pandemic sub-dataset ($0.4x10^{-2}$).

No evidence of recombination was found in total NA sequence datasets (results not shown). Based on this, it was assumed that all sites in the NA sequences of the human influenza virus subtype or lineage datasets shared a common evolutionary history, allowing them to be directly used in following SP analyses.

6.1.2 Overall Analysis of Selective Pressure

Global dN/dS values express the ratio of the rate of non-synonymous substitutions (dN) to the rate of synonymous substitutions (dS) over the entire coding region, providing a measure of the overall strength of selection acting on protein-coding genes [4].
Global dN/dS ratios for influenza NA gene in total time periods were similar across the different virus subtypes or lineages circulating among humans, ranging from 0.21 to 0.26 (Figure 6.4). Considering that a dN/dS ratio significantly less than 1 indicates negative SP (NSP) [4], these results evidenced that the different human influenza virus NA genes have been evolving under NSP of similar magnitude. The global dN/dS ratio for each human influenza virus NA gene was also very similar across the different time periods studied (temporal sub-datasets) (Figure 6.4), suggesting that neither the introduction of NAIs

into clinic (1999) and/or its increased use during 2009 A(H1N1) pandemic had an impact on the overall SP acting on NA gene.

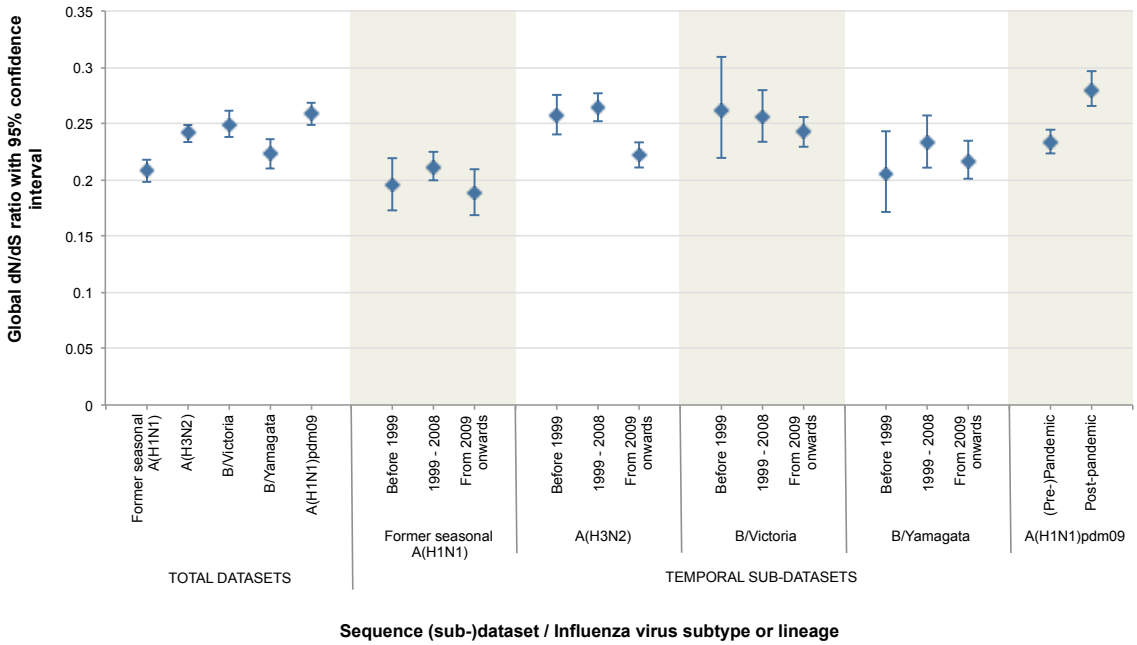


Figure 6.4 Global estimates of the dN/dS ratio in the neuraminidase gene of former seasonal A(H1N1), A(H3N2), B/Victoria and B/Yamagata-lineage, and A(H1N1)pdm09 viruses with 95% confidence intervals, in total and temporal sequence (sub-)datasets.

dN: rate of non-synonymous substitutions; dS: rate of synonymous substitutions

Analyses were conducted in HyPhy using the Muse-Gaut 94 model crossed with the model described as 012340, previously determined by Akaike's Information Criterion as the best time reversible model for influenza neuraminidase. Error bars show the 95% confidence interval.

6.1.3 Site-by-Site Analysis of Selective Pressure

6.1.3.1 Summary Results (SLAC and FEL consensus-based inference)

Table 6.2 summarizes the site-specific SP results obtained through consensus-based inference of Single-Likelihood Ancestor Counting (SLAC) and Fixed Effects Likelihood (FEL) individual results. When no consensus was verified, it was considered the result got by FEL method, except in the case of positively or negatively selected sites. These were only accepted under consensus.

Most sites (93.3% to 98.5%) on the NA gene of the different human influenza virus subtypes or lineages varied in their composition in total sequence dataset. From these variable sites, the majority (82.6% to 86.1%) was found to be experiencing a dN/dS ratio less than 1 that was statistically significant for approximately or slightly more than half of them – negatively selected sites (41.6% to 52.3%). For A(H3N2) subtype, the percentage of sites under significant purifying selection was exceptionally higher, reaching 67.3% of all variable sites. Approximately 13% to 17% of all variable sites, according to the virus subtype or lineage, were estimated to be experiencing a dN/dS ratio greater than 1. However, only for a very few of them, varying from 0.2% to 1.8%, this ratio was statistically significant - positive selection. A minor percentage of all variable sites within each human influenza virus NA were found under neutral evolution (dN/dS≈1; 0.7% to 2.0%).

Table 6.2 Summary characterization of the site-by-site selective pressure results obtained for the neuraminidase gene of former seasonal A(H1N1), A(H3N2), B/Victoria and B/Yamagata-lineage, and A(H1N1)pdm09 viruses, in total and temporal sequence (sub-)datasets.

	Influenza virus subtype or lineage/ sequence sub-dataset		Total codon sites [a]	PERCENTAGE (NUMBER) OF CODON SITES						SLAC and FEL consensus (%) [d]
				Variable [b]	dN/dS > 1 [c]	Positively selected [c]	dN/dS < 1 [c]	Negatively selected [c]	dN/dS ≈ 1 [c]	
TOTAL DATASETS	Former seasonal A(H1N1)		470	96.4 (453)	12.8 (58)	0.2 (1)	85.4 (387)	49.2 (223)	1.8 (8)	86.8
	A(H3N2)		469	98.5 (462)	13.0 (60)	1.1 (5)	86.1 (398)	67.3 (311)	0.9 (4)	92.1
	B/Victoria		466	96.4 (449)	16.3 (73)	1.8 (8)	82.6 (371)	51.9 (233)	1.1 (5)	87.1
	B/Yamagata		466	93.3 (435)	16.6 (72)	1.4 (6)	82.8 (360)	41.6 (181)	0.7 (3)	83.9
	A(H1N1)pdm09		469	97.0 (455)	14.9 (68)	1.3 (6)	83.1 (378)	52.3 (238)	2.0 (9)	83.4
TEMPORAL SUB-DATASETS	Former seasonal A(H1N1)	Before 1999	470	71.7 (337)	22.0 (74)	0.0 (0)	77.4 (261)	7.1 (24)	0.6 (2)	85.5
		1999-2008		93.4 (439)	15.3 (67)	0.2 (1)	83.8 (368)	35.5 (156)	0.9 (4)	84.5
		From 2009 onwards		75.1 (353)	19.8 (70)	0.0 (0)	78.8 (278)	13.6 (48)	1.4 (5)	86.6
	A(H3N2)	Before 1999	469	88.7 (416)	20.4 (85)	1.0 (4)	77.9 (324)	32.2 (134)	1.7 (7)	84.0
		1999-2008		96.8 (454)	15.4 (70)	1.1 (5)	82.6 (375)	46.9 (213)	2.0 (9)	85.5
		From 2009 onwards		96.2 (451)	12.4 (56)	0.9 (4)	86.5 (390)	53.2 (240)	1.1 (5)	90.4
	B/Victoria	Before 1999	466	39.7 (185)	30.3 (56)	0.0 (0)	69.7 (129)	3.2 (6)	0.0 (0)	94.0
		1999-2008		80.9 (377)	24.4 (92)	0.8 (3)	74.3 (280)	14.9 (56)	1.3 (5)	82.8
		From 2009 onwards		93.6 (436)	18.1 (79)	1.8 (8)	80.7 (352)	45.6 (199)	1.1 (5)	82.0
	B/Yamagata	Before 1999	466	44.6 (208)	26.0 (54)	0.0 (0)	74.0 (154)	4.8 (10)	0.0 (0)	94.8
		1999-2008		75.3 (351)	21.1 (74)	0.9 (3)	78.3 (275)	14.8 (52)	0.6 (2)	86.5
		From 2009 onwards		86.3 (402)	18.2 (73)	1.0 (4)	79.1 (318)	26.6 (107)	2.7 (11)	81.3
	A(H1N1)pdm09	(Pre-)pandemic	469	95.9 (450)	16.7 (75)	0.7 (3)	80.4 (362)	50.4 (227)	2.9 (13)	85.1
		Post-pandemic		93.8 (440)	18.0 (79)	0.7 (3)	79.5 (350)	33.2 (146)	2.5 (11)	82.3

dN: rate of non-synonymous substitutions; dS: rate of synonymous substitutions; SLAC: Single-Likelihood Ancestor Counting; FEL: Fixed Effects Likelihood.

[a] Stop codon not included; [b] Percentage relative to all codon sites; [c] Percentage relative to variable codon sites; [d] Percentage of codon sites with the same result for SLAC and FEL methods among all codon sites.

SLAC and FEL analyses were conducted in HyPhy using the Muse-Gaut 94 model crossed with the model described as 012340, previously determined by Akaike's Information Criterion as the best time reversible model for influenza neuraminidase.

(Footnotes Table 6.2 cont.)
The results were obtained throughout consensus-based inference of SLAC and FEL individual results. When no consensus was verified, it was considered the result got by FEL analysis, except in the case of positively or negatively selected sites that were only validated under consensus.

The splitting into different time periods revealed some differences in the distribution of the sites across the different SP profiles. However, these have to be interpreted carefully as most of them appear to be artefacts of the different size of temporal sub-datasets (see Table 6.1 above). This is clearly the case of the differences in the percentage of sites experiencing a dN/dS ratio less than 1 or under NSP observed for most or all human influenza virus subtypes or lineages (Table 6.2), as evidenced by the direct correlation of the variation and the number of sequences enclosed in the sub-datasets. The fact that the differences were more pronounced when the number of sequences was also more divergent further supports this. The increase in the percentage of positively selected sites in B/VIC lineage NA over the different time periods might also be an artefact of the increasingly larger size of sequence sub-datasets. However, this was the only virus subtype/lineage for which this increase was observed (Table 6.2).

6.1.3.2 Positively Selected Sites

6.1.3.2.1 Former seasonal influenza A(H1N1) virus neuraminidase

Site 275 was the only amino acid site in seasonal A(H1N1) virus NA under PSP during the total time period analysed (total sequence dataset) (Table 6.3). Structurally, site 275 is well-know to lie close to the enzymatic active site of N1 NA [6]. Moreover, it is the only NA site associated with clinical resistance to NAIs, specifically to oseltamivir (OS), and is also associated with highly reduced inhibition (HRI) by OS and peramivir (PER) *in vitro* (H275Y amino acid substitution) [7,8]. The detection of 275H/Y as the only amino acid polymorphism occurring at this site (Table 6.3), showed that PSP favoured the concerted substitution towards the amino acid residue conferring resistance or HRI - directional PSP.

Table 6.3 Codon sites under positive selective pressure in the neuraminidase gene of former seasonal A(H1N1), A(H3N2), B/Victoria and B/Yamagata-lineage, and A(H1N1)pdm09 viruses, in total and temporal sequence (sub-)datasets.

	Sequence (sub-)dataset		Total number	POSITIVELY SELECTED SITES: Codon site [a]	Structural domain and site particularities	SLAC: p value	SLAC: dN - dS scaled	SLAC: dN	SLAC: dS	FEL: p value	FEL: dN/dS	FEL: dN	FEL: dS	Amino acid polymorphism(s)
FORMER SEASONAL A(H1N1)	TOTAL DATASET		1	275 (274)	GH catalytic domain; Clinical R OS + HRI by OS and PER *in vitro* (NA H275Y)	0.0047	15.11	3.61	3.80	0.0057	2.25	0.56	4.02	61.6% H; ***38.4% Y***
	TEMPORAL SUB-DATASETS	Before 1999	0	-	-	-	-	-	-	-	-	-	-	-
		1999 - 2008	1	275 (274)	(see above)	0.00046	14.08	1.20	6.30	0.00014	3.38	0.27	12.60	76.1% H; ***23.9% Y***
		From 2009 onwards	0	-	-	-	-	-	-	-	-	-	-	-
A(H3N2)	TOTAL DATASET		5	44	Stalk domain	0.013	2.11	31.50	17.01	0.00020	2.56	2.48	0.97	97.7% S; 1.8% P; 0.3% F; 0.1% Y; 0.03% L
				148	GH catalytic domain, 150-cavity loop; Enhancement of the reduced NAI susceptibility conferred *in vitro* by E119V (NA T148I; synergy); N-glycosilation motif (N_{146})	2.45E-15	7.87	59.09	5.07	6.99E-12	9.02	4.17	0.46	96.0% T; ***2.7% I***; 1.0% K; 0.2% P; 0.05% A; 0.05% R
				151	GH catalytic domain, functional active site residue, 150-cavity loop; RI by OS (NA D151E) + (H)RI by ZA (NA D151A/G/V) *in vitro*	5.58E-15	8.88	67.01	6.09	6.33E-15	10.90	4.74	0.44	95.4% D; ***1.9% G***; 1.6% N; ***0.8% E***; ***0.2% V***; ***0.08% A***
				435	GH catalytic domain	0.011	1.33	11.63	2.47	0.051	3.35	0.82	0.24	93.1% E; 4.1% R; 2.2% K; 0.4% D; 0.2% G; 0.03% A
				468	GH catalytic domain	0.0083	1.31	12.00	3.00	0.017	3.50	0.74	0.21	99.2% P; 0.4% S; 0.2% H; 0.1% L; 0.03% R; 0.03% T
	TEMPORAL SUB-DATASETS	Before 1999	4	120	GH catalytic domain	0.0033	6.08	9.50	1.00	0.0027	9.02	3.25	0.36	95.9% P; 3.5% L; 0.6% H
				370	GH catalytic domain	0.0062	4.52	7.02	0.71	0.015	7.24	2.59	0.36	68.1% L; 19.1% S; 12.8% F
				432	GH catalytic domain, 430-cavity loop	0.055	3.18	4.44	0.00	0.016	3.24E+08	1.62	0.00	98.9% Q; 0.3% E; 0.3% K; 0.2% P; 0.3% L
				434	GH catalytic domain	0.039	2.86	4.00	0.00	0.013	inf	1.36	0.00	97.9% T; 1.5%N; 0.2% I; 0.5% A
		1999 - 2008	5	44	(see above)	0.022	3.06	15.00	6.00	0.0023	3.36	2.70	0.80	97.8% S; 1.9% P; 0.2% F; 0.05% Y
				147	GH catalytic domain, 150-cavity loop; Epitope contacting residue (Mem5 antibody) with potential effect on antibody binding	0.018	2.41	8.30	1.20	0.0021	9.44	1.44	0.15	87.8% D; 11.8% N; 0.3% Y; 0.1% G
				148	(see above)	1.60E-05	6.39	21.85	3.04	0.00042	5.22	3.61	0.69	97.0% T; ***2.1% I***; 0.8% K; 0.05% P; 0.05% A
				151	(see above)	5.25E-09	11.96	38.87	3.69	4.56E-10	11.62	6.82	0.59	94.4% D; ***2.3% G***; 1.8% N; ***1.2% E***; ***0.3% V***; ***0.05% A***
				435	(see above)	0.022	1.96	5.76	0.00	0.020	inf	0.98	0.00	98.8% E; 0.6% D; 0.5% K; 0.05% G
		From 2009 onwards	4	40	Stalk domain	0.043	2.13	8.95	2.43	0.012	4.71	1.73	0.37	94.6% Y; 3.1% C; 2.1% H; 0.1% F; 0.05% D; 0.05% S
				148	(see above)	1.65E-11	11.95	38.55	2.05	3.90E-09	14.68	5.91	0.40	95.0% T; ***3.4% I***; 1.2% K; 0.2% P; 0.05% A; 0.1% R
				151	(see above)	6.00E-08	10.10	34.48	3.64	3.55E-08	9.60	5.31	0.55	95.8% D; 1.8% N; ***1.7% G***; ***0.4% E***; ***0.1% V***; ***0.1% A***
				468	(see above)	0.0034	2.29	7.00	0.00	0.00095	1.02E+06	1.02	0.00	99.0% P; 0.6% S; 0.2% H; 0.1% L; 0.05% T

(Table 6.3 cont.)

	Sequence (sub-)dataset		Total number	Codon site [a]	Structural domain and site particularities	POSITIVELY SELECTED SITES								Amino acid polymorphism(s)
						SLAC				FEL				
						p value	dN - dS scaled	dN	dS	p value	dN/dS	dN	dS	
B/VICTORIA	TOTAL DATASET		8	46 (47)	Stalk domain	0.0070	2.31	8.48	1.00	0.010	7.12	1.11	0.16	96.4% T; 3.3% I; 0.2% N; 0.05% S; 0.05% A; 0.05% P
				48 (49)	Stalk domain	0.0023	2.32	7.51	0.00	0.0055	inf	0.99	0.00	99.1% P; 0.6% S; 0.1% Q; 0.1% T; 0.05% R; 0.05% L
				65 (66)	Stalk domain; N-glycosilation motif (N_{64})	0.018	3.58	22.81	11.19	0.052	1.86	3.27	1.76	90.8% R; 7.7% H; 0.7% L; 0.3% C; 0.3% S; 0.2% Y
				68 (69)	Stalk domain	0.017	1.54	5.00	0.00	0.024	inf	0.65	0.00	98.8% T; 1.2% A; 0.05% K
				72 (73)	GH catalytic domain	0.00057	3.38	11.96	1.01	0.0091	6.96	1.57	0.23	98.3% T; 1.3% I; 0.3% A; 0.2%K; 0.05% S
				73 (74)	GH catalytic domain	6.46E-05	5.64	22.48	4.19	0.00025	4.72	3.09	0.65	67.7% L; 32.0% F; 0.2% P; 0.1% S; 0.05% R; 0.05% I
				395 (390)	GH catalytic domain; RI by OS and PER *in vitro* (NA A395E)	3.16E-06	7.55	30.48	6.01	4.70E-05	4.26	4.03	0.95	88.7% A; 5.7% T; 4.1% V; 1.3% D; 0.2% I; ***0.1% E***
				465 (465)	GH catalytic domain	0.019	1.85	7.00	1.00	0.028	5.82	0.90	0.15	94.8% A; 5.0% T; 0.05% D; 0.05% G; 0.05% V
	TEMPORAL SUB-DATASETS	Before 1999	0	-	-	-	-	-	-	-	-	-	-	-
		1999 - 2008	3	42 (43)	Stalk domain	0.036	4.89	4.15	0.00	0.039	inf	2.15	0.00	80.5% P; 16.4% S; 1.6% Q; 1.0% R; 0.5% L
				65 (66)	(see above)	0.031	6.37	6.42	1.02	0.029	5.91	3.53	0.60	88.3% R; 10.9% H; 0.5% L; 0.3% C
				395 (390)	(see above)	0.0077	7.08	6.00	0.00	0.0031	inf	3.11	0.00	92.2% A; 5.7% T; 1.8% V; 0.3% D
		From 2009 onwards	8	46 (47)	(see above)	0.012	2.39	5.50	0.00	0.0058	1.79E+16	1.00	5.55E-17	99.0% T; 0.8% I; 0.1% N; 0.06% A; 0.06% P
				48 (49)	(see above)	0.039	1.74	4.00	0.00	0.042	inf	0.74	0.00	99.4% P; 0.4% S; 0.1% T
				54 (55)	Stalk domain	0.016	2.50	5.75	0.00	0.0039	inf	1.28	0.00	99.0% C; 0.9% Y; 0.06% R; 0.06% F
				68 (69)	(see above)	0.039	1.74	4.00	0.00	0.044	inf	0.72	0.00	98.8% T; 1.1% A; 0.06% K
				72 (73)	(see above)	0.00085	4.53	11.45	1.01	0.012	6.66	2.09	0.31	97.9% T; 1.6% I; 0.3% A; 0.06%K; 0.06% S
				73 (74)	(see above)	0.00016	6.31	16.63	2.09	0.00036	6.96	3.20	0.46	63.8% L; 35.8% F; 0.1% S; 0.06% P; 0.06% R; 0.06% I
				199 (200)	GH catalytic domain	0.017	2.44	5.62	0.00	0.0057	inf	1.16	0.00	82.2% N; 17.4% D; 0.2% K; 0.06% S; 0.06% G
				395 (390)	(see above)	0.00010	7.80	22.98	5.01	0.00082	3.79	4.18	1.10	87.8% A; 5.6% T; 4.8% V; 1.5% D; 0.2% I; ***0.1% E***
B/YAMAGATA	TOTAL DATASET		6	42 (43)	Stalk domain	0.0058	5.35	15.28	4.01	0.014	3.32	3.31	1.00	46.8% Q; 41.9% R; 9.2% P; 1.4% T; 0.3% S; 0.2% L; 0.2% K
				65 (66)	Stalk domain; N-glycosilation motif (N_{64})	0.0015	5.18	12.92	2.03	0.0032	5.47	2.71	0.50	88.5% R; 10.8% H; 0.4% L; 0.1% S; 0.1% C
				73 (74)	GH catalytic domain	0.0070	4.03	10.48	2.01	0.018	4.23	2.07	0.49	90.4% L; 8.0% P; 1.6% F
				106 (110-111[b])	GH catalytic domain	0.028	2.60	6.49	1.00	0.012	7.26	1.25	0.17	65.9% T; 32.3% I; 1.6% N; 0.2% A; 0.07% V
				198 (199)	GH catalytic domain	0.00036	6.13	14.05	1.16	0.00091	9.93	2.86	0.29	93.8% S; 6.2% N
				465 (465)	GH catalytic domain	0.010	3.33	8.00	1.00	0.020	6.18	1.51	0.24	58.6% A; 41.0% T; 0.1% N; 0.1% V; 0.07% D; 0.07% S
	TEMPORAL SUB-DATASETS	Before 1999	0	-	-	-	-	-	-	-	-	-	-	-
		1999 - 2008	3	**65 (66)**	(see above)	0.014	9.14	7.48	1.01	0.018	6.47	4.78	0.74	93.5% R; 5.3% H; 0.5% L; 0.5% S; 0.2% C
				198 (199)	(see above)	0.025	8.94	7.49	1.16	0.039	5.29	4.62	0.87	94.7% S; 5.3% N
				395 (390)	GH catalytic domain; RI by OS and PER *in vitro* (NA A395E)	0.039	5.65	4.00	0.00	0.022	inf	2.28	0.00	94.7% A; 3.9% T; 1.0% S; 0.5% V
		From 2009 onwards	4	**65 (66)**	(see above)	0.019	4.05	4.96	0.00	0.016	2.91E+14	1.52	5.23E-15	85.8% R; 14.0% H; 0.1% L; 0.1% C
				106 (110-111[b])	(see above)	0.0080	4.89	5.98	0.00	0.0010	inf	2.06	0.00	49.2% I; 48.0% T; 2.4% N; 0.3% A; 0.1% V
				198 (199)	(see above)	0.0060	5.75	7.03	0.00	0.0046	inf	2.41	0.00	93.0% S; 7.0% N
				465 (465)	(see above)	0.017	4.09	5.00	0.00	0.011	inf	1.69	0.00	50.4% T; 49.2% A; 0.2% N; 0.1% V; 0.1% S

(Table 6.3 cont.)

	Sequence (sub-)dataset		Total number	Codon site [a]	Structural domain and site particularities	POSITIVELY SELECTED SITES								Amino acid polymorphism(s)
						SLAC				FEL				
						p value	dN - dS scaled	dN	dS	p value	dN/dS	dN	dS	
A(H1N1)pdm09	TOTAL DATASET		6	46 (44)	Stalk domain	0.014	1.75	11.99	3.28	0.0047	4.24	1.27	0.30	98.5% I; 1.1% T; 0.4% V; 0.03% M
				74 (72)	Stalk domain	0.013	1.52	8.78	1.20	0.0026	9.03	1.00	0.11	99.2% F; 0.5% L; 0.3% V; 0.03% I; 0.03% S
				95 (95)	GH catalytic domain; Epitope contacting residue (CD6 antibody) crucial for antibody binding	0.054	1.32	10.16	3.60	0.031	3.19	1.06	0.33	98.7% S; 0.8% N; 0.4% G; 0.06% R; 0.06% I
				247 (246)	GH catalytic domain; RI by OS *in vitro* (NA S247G); Enhancement of the (H)RI by OS and PER conferred *in vitro* by H275Y (NA S247N; synergy)	0.0075	1.93	12.00	2.40	0.0026	5.65	1.26	0.22	99.0% S; ***0.9% N***; ***0.03% G***; 0.03% I
				275 (274)	GH catalytic domain; Clinical R OS + (H)RI by OS and PER *in vitro* (NA H275Y)	0.00016	5.42	44.43	17.49	7.25E-06	3.01	3.97	1.32	95.7% H; ***4.3% Y***
				450 (451)	GH catalytic domain; Epitope contacting residue (CD6 antibody)	0.053	0.84	4.15	0.00	0.011	inf	0.43	0.00	99.7% S; 0.2% G; 0.03% I
	TEMPORAL SUB-DATASETS	(Pre-) Pandemic period	3	46 (44)	(see above)	0.013	2.12	8.14	1.10	0.0038	8.59	1.22	0.14	98.9% I; 0.9% T; 0.2% V
				74 (72)	(see above)	0.040	1.38	4.60	0.00	0.0052	inf	0.75	0.00	99.5% F; 0.4% L; 0.1% V; 0.03% I
				275 (274)	(see above)	1.50E-06	8.33	33.58	5.86	1.61E-09	7.95	4.88	0.61	96.9% H; ***3.1% Y***; 0.03% R
		Post-pandemic period	3	247 (246)	(see above)	0.0035	3.63	7.90	0.00	0.00022	inf	2.06	0.00	98.3% S; ***1.6% N***; 0.1% I
				275 (274)	(see above)	0.0038	6.96	24.42	9.24	5.44E-05	3.77	5.82	1.55	94.2% H; ***5.8% Y***
				454 (455)	GH catalytic domain	0.018	3.21	8.99	2.00	0.0097	4.83	2.05	0.42	98.5% G; 0.7% S; 0.7% D; 0.1% V

SLAC: Single-Likelihood Ancestor Counting; FEL: Fixed Effects Likelihood; dN: rate of non-synonymous substitutions; dS: rate of synonymous substitutions; GH: Globular head; R: Resistance; HRI: Highly reduced inhibition; RI: Reduced inhibition; (H)RI: HRI or RI; OS: Oseltamivir; PER: Peramivir; ZA: Zanamivir; NAI: Neuraminidase inhibitor; inf: infinity (dS=0.00)

[a] Standard N2 numbering is indicated in brackets after the type or subtype-specific numbering; [b] Precise N2 numbering cannot be given as influenza B NA carries a deletion in the alignment compared to A(H3N2) NA

Positive selection was only considered when detected by both SLAC and FEL methods (consensus-based criterion). Sites under positive selective pressure in more than one temporal sub-dataset are highlighted in bold and italic, as well as the amino acid residues associated with NAI resistance or (H)RI among the amino acid polymorphism(s) identified in the site. The location of the NA structural domains was based on Colman [9] and Wei [10] (former seasonal N1); Colman [9], Colman and Ward [11] and Air and Laver [12] (N2); Colman and Ward [11] and Flandorfer *et al.* [13] (influenza B NA); and Colman [9] and da Silva *et al.* [14] (2009 pandemic N1). Site particularities were defined according to the information found at Li *et al.* [15] (cavity loops); Colman [9] (N-glycosylation motifs); WHO [8] and WHO [16] (association with NAI resistance or (H)RI); and Venkatramani *et al.* [17] and Wan *et al.* [18] (epitope contacting residues).

Temporal splitting revealed that site 275 was only under PSP between 1999 and 2008, corresponding to the period after NAI introduction into clinic but before its increased use during 2009 pandemic. In fact, the H275Y amino acid substitution favoured by positive selection was not detected in any of the sequences from before 1999, when NAIs were not available in the market (100% H275), and, from 2009 onwards was already fixed in the seasonal A(H1N1) virus population (3.6% H275; 96.4% 275Y). No other site besides 275 was found under PSP in the different time periods studied.

Given the emergence and worldwide spread of a H275Y OS-resistant variant since late 2007, two additional time periods were studied for seasonal A(H1N1) subtype to further investigate the PSP acting on site 275 between 1999 and 2008. Specifically, (1) 1999 to 2006, and (2) 1999 to 2007. Evidence for positive selection was only obtained in the latter period (SLAC: p =0.0028; dN-dS scaled=8.33; dN=8.33; dS=0.00; FEL: p=0.0014; dN/dS=infinite; dN=2.82; dS=0.00), suggesting that it may have only started in 2007 and thereby may be simply an artefact of the worldwide spread of the drug-resistant variant.

6.1.3.2.2 Influenza A(H3N2) virus neuraminidase

Sites 44, 148, 151, 435 and 468 in human influenza N2 NA were under PSP during the total time period analysed. Sites 148 and 151 were both under strong PSP, as evidenced by the extremely low p-values calculated by SLAC and FEL methods (10^{-12} to 10^{-15}) (Table 6.3). Structurally, these sites are known to be located very near (site 148 - 150-cavity loop) [15] or at the NA active site (site 151 - functional residue) [19]. Moreover, they are both associated with (H)RI by NAI drugs *in vitro*. Several amino acid substitutions at site 151 are known to confer (H)RI (D151E/V/A/G) [8], while the T148I amino acid substitution at site 148 has a synergistic effect on the reduced susceptibility caused by NA E119V substitution [16]. Site 148 is additionally involved in the potential N-linked glycosylation of Asn146 [9]. Two other positively selected sites – sites 435 and 468, belong to the globular head catalytic domain, but are located distantly from the active site (Figure 6.5).

All five positively selected sites presented an amino acid profile highly polymorphic, with the different variants occurring at very low frequency (0.03% to 4.1%) (Table 6.3). This indicated that positive selection favoured the maintenance of several amino acid variants

at these sites in virus population, prompting diversity – diversifying PSP. All amino acid substitutions at site 151 known to confer (H)RI were among the polymorphisms identified (D151E/V/A/G), as well as the synergistic T148I amino acid substitution at site 148 (Table 6.3). However, only in one sequence from 2011 this synergistic substitution was found together with the NA E119V amino acid substitution conferring (H)RI.

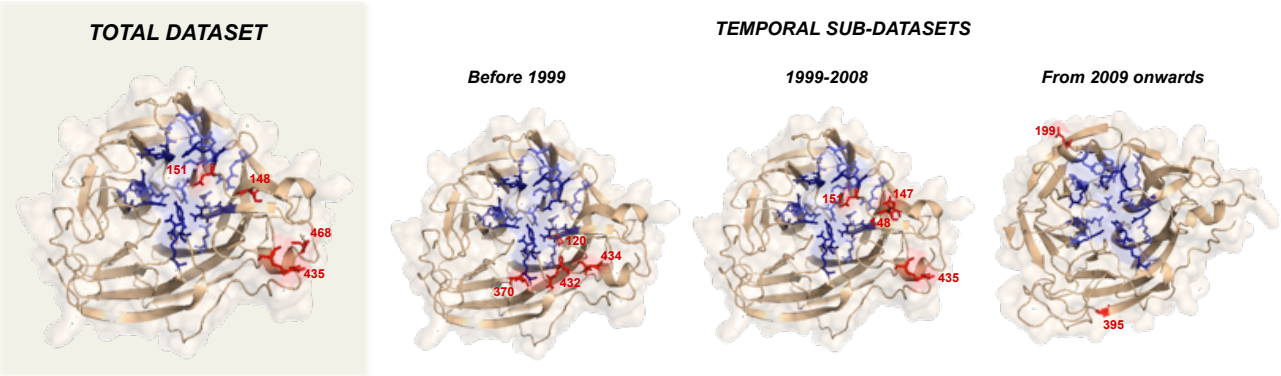


Figure 6.5 Mapping of the human influenza N2 neuraminidase sites under positive selective pressure onto the three-dimensional structure of the protein globular head domain.

The figures were generated in PyMOL, using the structure file of the neuraminidase (NA) globular head domain from the A(H2N2) virus A/Tokyo/3/1967, previously retrieved from the RCSB Protein Data Bank (PDB ID: 1NN2). The globular head domain is presented in the same orientation for all different time periods. The amino acid residues forming the NA active site (based on Colman *et al.* [19]) are coloured in dark blue (functional residues) or light purple (framework residues) and shown as sticks, while positively selected sites are highlighted as red sticks.

Temporal splitting showed that sites 148 and 151 were continually under PSP after the introduction of NAI drug class into clinic (between 1999 and 2008, and from 2009 onwards) (Table 6.3). It also showed that PSP at site 148 was stronger during the period of increased NAI drug use (from 2009 onwards), as evidenced by the notably lower p-value and greater dN-dS scaled (SLAC) or dN/dS (FEL) value, compared to 1999 to 2008 (Table 6.3). At amino acid level, this stronger PSP translated into a new polymorphism (T148R) and a slight increase in frequency for three variants (T148I/K/P). Other positively selected sites located near or at the NA active site included site 120 but only during the period before 1999 (no NAI drug available), and site 147 between 1999 and 2008 (Figure 6.5). Site 147 is a known epitope contacting residue [17], being under selection of the host´s antibody-mediated immune response.

6.1.3.2.3 Influenza B/Victoria-lineage neuraminidase

Positively selected sites in influenza B/VIC-lineage NA included sites 46, 48, 65, 68, 72, 73, 395 and 465, in the total time period. The first 4 sites are located within the stalk domain of the protein, while the last 4 belong to the catalytic globular head domain (Table 6.3). None of these latter sites lie near the enzymatic active site, as evidenced by structural mapping (sites 395 and 465; Figure 6.6) or based on its location at the beginning of the domain (sites 72 and 73; not included in the NA structure files available for use). Positively selected site 395 is associated with reduced inhibition (RI) by OS and PER *in vitro* (NA A395E amino acid substitution) [8].

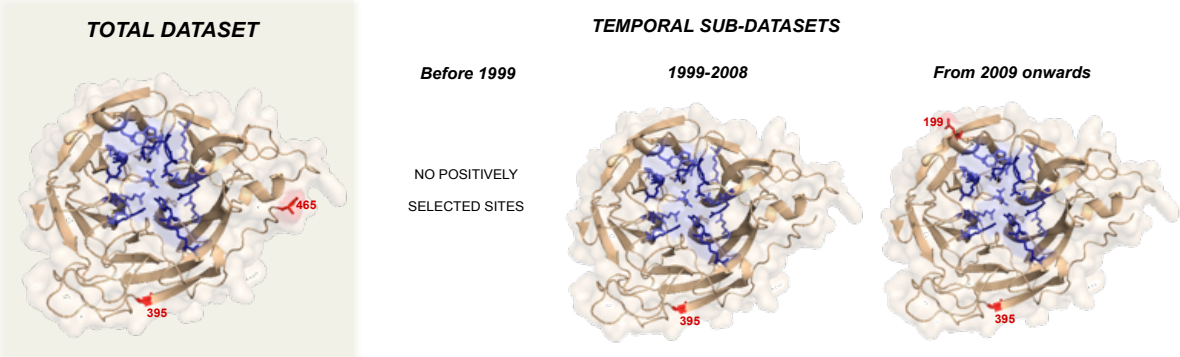


Figure 6.6 Mapping of the influenza B/Victoria-lineage neuraminidase sites under positive selective pressure onto the three-dimensional structure of the protein globular head domain.

The figures were generated and annotated as described in Figure 6.5, using the structure file of the neuraminidase globular head domain from the B/Victoria-lineage vaccine virus B/Brisbane/60/2008 (PDB ID: 4CPL; only one globular head of the two described in the file is shown). Positively selected sites 72 and 73 (total time period and from 2009 onwards) could not be indicated as are missing in the structure file used (amino acid residues 77 to 466).

All positively selected sites except site 68 were highly polymorphic, presenting 4 to 5 different amino acid variants (Table 6.3). Site 68 presented two different variants. Moreover, all amino acid polymorphisms occurred at very low frequency (0.05% to 7.7%), suggesting that PSP prompted the maintenance of amino acid diversity - positive diversifying selection. The only exception was site 73, in which the L73F amino acid substitution occurred at a much higher frequency (32.0%) than all other variants (Table 6.3), indicating that PSP favoured the substitution towards the phenylalanine (F) residue

– positive directional selection. The A395E amino acid substitution known to confer RI was one of the polymorphisms found at site 395.

None of the sites under PSP between 1999 and 2008 and from 2009 onwards is also located near or at the NA active site, lying in the stalk domain or in the globular head domain but at either its beginning (sites 72 and 73) or surface region (sites 199 and 395; Figure 6.6) (Table 6.3). Site 395 associated with NAI RI was continually under PSP during these two time periods, with the site-specific values calculated by both SLAC and FEL methods suggesting similar PSP in the two periods (Table 6.3). The amino acid variant conferring RI only emerged during the period of increased NAI drug use (A395E; from 2009 onwards). It is important to note that the lack of positively selected sites before 1999 might be due to the extremely low number of sequences comprising the sub-dataset (N=34; see Table 6.1 above).

6.1.3.2.4 Influenza B/Yamagata-lineage neuraminidase

Six sites in influenza B/YAM-lineage NA were under PSP during the total time period analysed. Specifically, sites 42, 65, 73, 106, 198 and 465 (Table 6.3). Most of these sites are located within the globular head catalytic domain, but only site 198 lies near the enzymatic active site, as evidenced in Figure 6.7 (site 73 not shown as is missing in the structure file used, but lies at the beginning of the domain). Site 198 was also the most strongly selected site, based on the simultaneously lowest p-value and greatest dN-dS scaled (SLAC) or dN/dS (FEL) value (Table 6.3).

Most positively selected sites were highly polymorphic, presenting 4 to 6 different amino acid variants. Sites 73 and 198 were the only exceptions, presenting two and a single amino acid variant, respectively (Table 6.3). However, all sites appeared to be under positive directional selection, based on the much higher frequency at which one of the variants within each polymorphic site was detected (Q42R; R65H; L73P; T106I; A465T). Only for site 73, the frequency at which the different variants were detected was not so distinct, making the situation less clear.

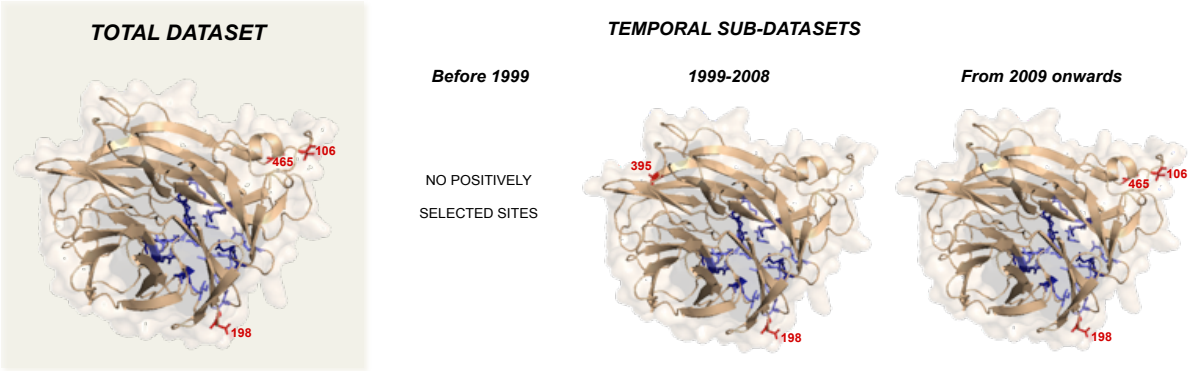


Figure 6.7 Mapping of the influenza B/Yamagata-lineage neuraminidase sites under positive selective pressure onto the three-dimensional structure of the protein globular head domain.

The figures were generated and annotated as described in Figure 6.5, using the structure file of the neuraminidase globular head domain from the B/Yamagata-lineage reference virus B/Perth/211/2001 (PDB ID: 3K36; only one globular head of the two described in the file is shown). Positively selected site 73 (total time period) could not be indicated as is missing in the structure file used (amino acid residues 77 to 466).

Temporal splitting showed that sites 65 and 198 were continually under PSP after the licensing of NAI drugs (between 1999 and 2008, and from 2009 onwards) (Table 6.3). Only site 198 lies, however, near the active site of the protein (drug target), as above-mentioned. Also, there was no consensus for a particular change in the strength of PSP acting at these sites between the two time periods (SLAC – decrease; FEL – increase). Site 395 associated with NAI RI, as already mentioned for B/VIC lineage, but located distantly from the NA active site, was under PSP between 1999 and 2008. Nevertheless, the A395E amino substitution known to confer RI was not among the polymorphisms identified (Table 6.3). Similarly to B/VIC lineage, it is important to note that the lack of positively selected sites before 1999 might be due to the low number of sequences comprising the sub-dataset (N=83; see Table 6.1 above).

6.1.3.2.5 2009 pandemic influenza A(H1N1) virus neuraminidase

Sites 46, 74, 95, 247, 275 and 450 in 2009 pandemic N1 NA were under PSP during the total time period analysed. Most of these sites are located within the globular head catalytic domain (Table 6.3), but only sites 247 and 275 lie close to the enzymatic active site (Figure 6.8). As above-mentioned for seasonal A(H1N1) subtype, site 275 is the only NA site associated with clinical resistance to OS. Moreover, in A(H1N1)pdm09 subtype, it

is associated with (H)RI by OS and PER *in vitro* (H275Y amino acid substitution) [8]. Site 247 is also associated with NAI (H)RI, specifically with RI by OS *in vitro* (NA S247G amino acid substitution) and with an enhancement of the H(RI) conferred by H275Y substitution (NA S247N amino acid substitution; synergistic effect) [16]. Positively selected sites 95 and 450 lie at the surface of the globular head (Figure 6.8), which is in agreement with their known role of epitope contacting residue [18]. Antibody-mediated immune selection contributed therefore for the PSP acting at both sites.

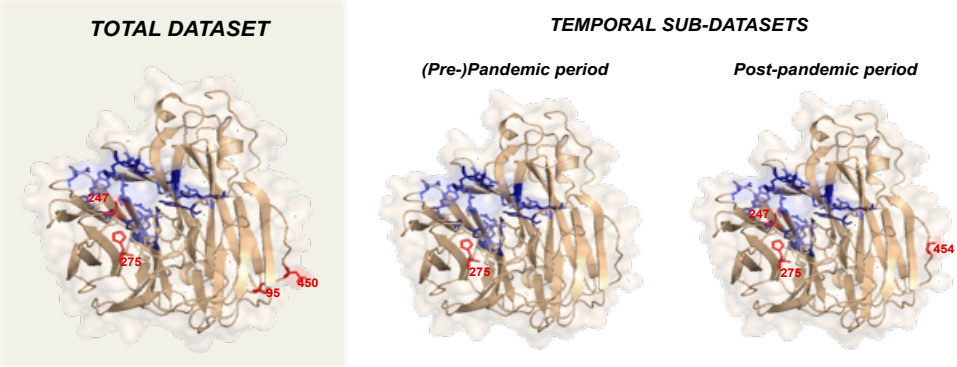


Figure 6.8 Mapping of the influenza 2009 pandemic N1 neuraminidase sites under positive selective pressure onto the three-dimensional structure of the protein globular head domain.

The figures were generated and annotated as described in Figure 6.5, using the structure file of the neuraminidase globular head domain from the A(H1N1)pdm09 reference virus A/California/04/2009 (PDB ID: 3NSS; only one globular head of the two described in the file is shown).

The 275H/Y was the only amino acid polymorphism detected at site 275 (Table 6.3), evidencing that positive directional selection was operating at the site, favouring the substitution towards the amino acid residue conferring resistance or (H)RI. All other positively selected sites presented 2 to 4 different amino acid variants at very low frequencies (0.03% to 1.1%), suggesting positive diversifying selection. The RI-conferring S247G and the synergistic S247N amino acid substitutions were among the polymorphisms detected at site 247. However, this latter was never found in combination with NA H275Y.

Site 275 was under PSP in both (pre-)pandemic and post-pandemic period, with SLAC and FEL site-specific values suggesting a stronger PSP in the former period, during which NAI drugs were more widely used (lower p-value; greater dN-dS scaled or dN/dS value)

(Table 6.3). Site 247 was otherwise only positively selected during the post-pandemic period. None of the other sites under PSP in the two time periods is located near the active site, lying either at the stalk domain (sites 46 and 74) or at the surface of the globular head domain (site 454; Figure 6.8).

6.1.3.3 Selective Pressure Profile of the Sites Associated with Resistance or (Highly) Reduced Inhibition by Neuraminidase Inhibitors and/or Contacting with the Drug (Active Site)

Most amino acid substitutions conferring resistance or (H)RI by NAI drugs or having a synergistic effect on the level of inhibition caused are influenza type- or subtype-specific. The SP profile of the sites at which these substitutions occur and of further sites contacting directly or indirectly with the drug (NA active site), are therefore showed separately by influenza NA type or subtype (N1, N2, and influenza B) in Figure 6.9 (A, B, and C, respectively).

N1 NA sites from either former or current circulating A(H1N1) subtypes associated with NAI resistance or (H)RI and/or contacting with the drug were essentially under negative selection (majority) or experiencing a not significant (n.s.) dN/dS<1 during the total time period. In fact, several sites presented the same profile in both subtypes, particularly those under NSP (Figure 6.9A). Site 275 was also under PSP in both subtypes, as detailed above in sections 6.1.3.2.1 (seasonal A(H1N1)) and 6.1.3.2.5 (A(H1N1)pdm09). Site 247 associated with NAI (H)RI in exclusively A(H1N1)pdm09 subtype was also under PSP (detailed in section 6.1.3.2.5). Few sites were found to be experiencing an n.s. dN/dS>1, including sites 70, 117 and 155 in seasonal N1 NA, and site 199 in 2009 pandemic N1 NA (Figure 6.9A). The single or at least one of the amino acid substitutions at these sites known to confer or enhance (H)RI were among the polymorphism(s) identified (N70S, I117M, Y155H; D199G/N)(presented below in Figure 6.11A). Temporal splitting revealed that seasonal N1 NA sites 119 and 228 maintained the same SP profile across the different time periods. All other sites in seasonal N1 NA experienced varied profiles but with no similar or particular trend that could be associated with the differences in NAI drug use defined by the different time periods (Figure 6.9A). It is possible that the different size of sequence sub-datasets may be contributing for some of the variations observed, as evidenced by the higher number of conserved sites before 1999 and from

2009 onwards (smaller sub-datasets), and the higher similarity of the 1999 to 2008 and total time period SP profiles (Figure 6.9A). Also, the very short time period covered from 2009 onwards (2 years), due to the rapid replacement by the new pandemic virus, may have contributed for the conserved status of several sites. In A(H1N1)pdm09 subtype, most sites maintained the same SP profile in both (pre-) pandemic and post-pandemic periods (Figure 6.9A). Also, the variations observed involved essentially a change between significant and n.s. dN/dS<1. Only for sites 136 and 151 the change was more pronounced, from significant or n.s. dN/dS<1 to n.s. dN/dS>1.

A - N1 NEURAMINIDASE

Codon site		Characteristics	FORMER SEASONAL A(H1N1)				A(H1N1)pdm09		
			TOTAL DATASET	TEMPORAL SUB-DATASETS			TOTAL DATASET	TEMPORAL SUB-DATASETS	
				Before 1999	1999 - 2008	From 2009 onwards		(Pre-)pandemic period	Post-pandemic period
70		↓ RI ZA (N1)					-	-	-
117		↓ E119V ZA; N295S+H275Y OS+ZA (N1)					-	-	-
118	■								
119	□	↓ (H)RI OS+ZA+PER (N1+N1pdm09) / (H)RI LAN (N1pdm09) ↓ H275Y PER (N1pdm09)	*				*		*
136		↓ (H)RI ZA+PER (N1+N1pdm09) / RI LAN (N1pdm09)	*		*				
151	■	↓ RI ZA+PER (N1pdm09) ↓ H275Y OS+ZA+PER (N1)	*						
152	■			*					
155		↓ (H)RI OS+ZA+PER (N1)					-	-	-
156	□								
179	□								
180	□								
199	□	↓ RI OS (N1pdm09) ↓ H275Y OS (N1pdm09)							*
223	□	↓ RI OS+ZA+PER (N1pdm09) ↓ H275Y PER; N295S+H275Y OS+ZA (N1) / H275Y ZA+PER (N1pdm09)		*					
225	■								
228	□					*			
247		↓ RI OS (N1pdm09) ↓ H275Y OS+ZA+PER (N1pdm09)	-	-	-	-			
275		⊖ R OS; (H)RI OS+PER (N1+N1pdm09)		*					
277	■			*					
278	□								
293	■	↓ RI ZA (N1)				*			
295	□	↓ (H)RI OS+PER (N1) / HRI OS; RI PER (N1pdm09)							
313 [a]		↓ RI OS+ZA (N1pdm09)	-	-	-	-			*
325		↓ RI OS (N1pdm09)	-	-	-	-			*
368	■				*				
402	■						*		
425	□								
427 [a]		↓ RI OS+ZA (N1pdm09)	-	-	-	-			

B - N2 NEURAMINIDASE (A(H3N2))

Codon site		Characteristics	TOTAL DATASET	TEMPORAL SUB-DATASETS		
				Before 1999	1999 - 2008	From 2009 onwards
41		↓ RI OS			*	*
118	■					
119	□	↓ (H)RI OS; RI ZA	*	*		
136		↓ (H)RI ZA				
148		↓ E119V OS+ZA+PER+LAN				
151	■	↓ RI OS; (H)RI ZA		*		
152	■					
156	□					
178	□					
179	□					
198	□					

B *(cont.)* - N2 NEURAMINIDASE (A(H3N2))

Codon site		Characteristics	TOTAL DATASET	TEMPORAL SUB-DATASETS		
				Before 1999	1999 - 2008	From 2009 onwards
222	□	↓ RI OS		*		
224	■	↓ (H)RI OS; RI ZA				
226		↓ RI OS		*		
227	□					
276	■	↓ RI OS; HRI ZA				
277	□					
292	■	↓ (H)RI OS+ZA+PER			*	
294	□	↓ HRI OS				
371	■	↓ RI OS+ZA			*	
406	■					
425	□			*		

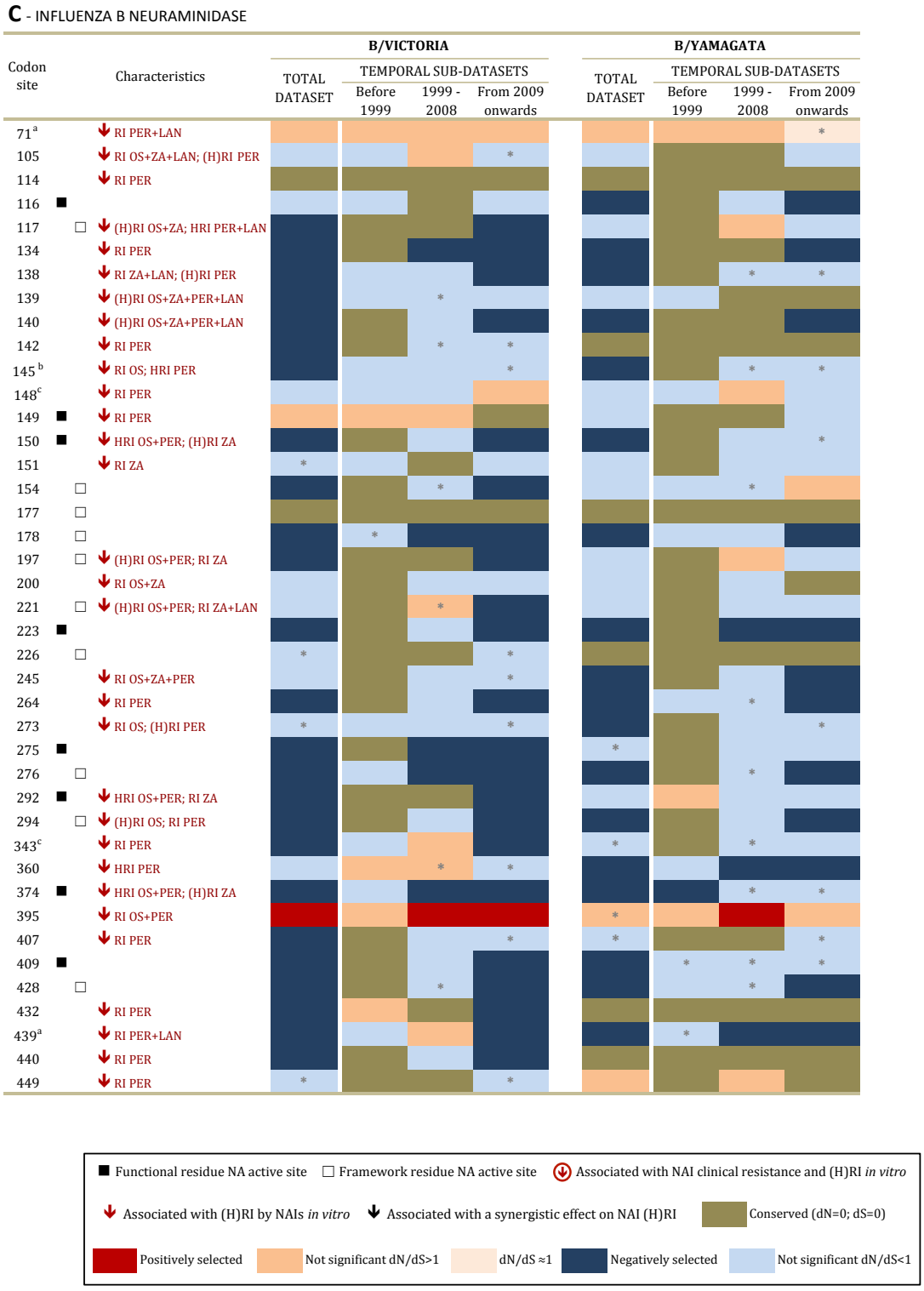


Figure 6.9 Selective pressure profile of the sites associated with neuraminidase inhibitor resistance or (highly) reduced inhibition and/or contacting with the drug (active site) in human influenza N1 (**A**), N2 (**B**) and B (**C**) neuraminidase, in total and temporal sequence (sub-)datasets.

NA: Neuraminidase; NAI: NA inhibitor; RI: Reduced inhibition; HRI: Highly reduced inhibition; (H)RI: RI or HRI; OS: Oseltamivir; ZA: Zanamivir; PER: Peramivir; LAN: Laninamivir

(Footnotes Figure 6.9 cont.)
Site-specific selective pressure profiles were determined through consensus-based inference of Single-Likelihood Ancestor Counting (SLAC) and Fixed Effects Likelihood (FEL) individual results. When no consensus was verified, it was considered the result got by FEL method, except in the case of positively or negatively selected sites that were only validated under consensus. Individual results are indicated with an asterisk (*). Functional and framework NA active site residues were defined according to Colman *et al.* [19], while the association to NAI resistance or (H)RI was based on WHO [8] and WHO [16]. **Panel A**: [a] Only when combined with each other (Q313K+I427T), individual phenotype not known; **Panel C**: [a] Only when combined with each other (V71A+H439R), individual phenotype not known; [b] Only in combination with site 142 (Y142H+G145R), individual phenotype of G145R amino acid substitution not known; [c] Only when combined with each other (G148R+K343E), individual phenotype not known.

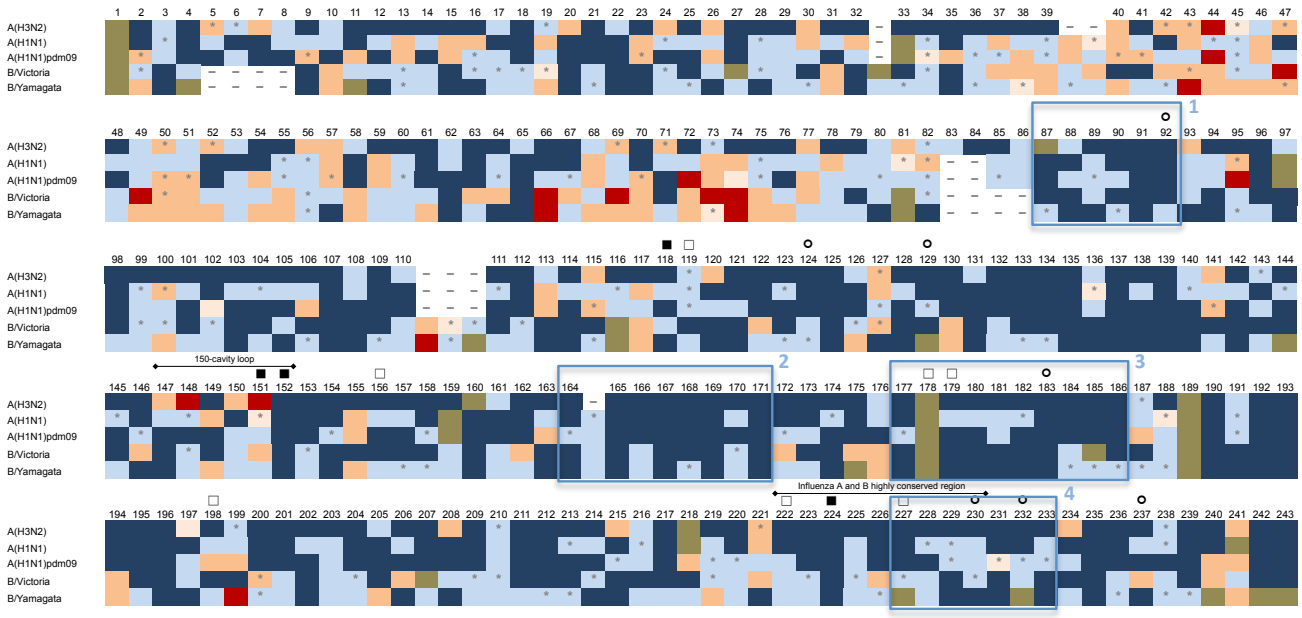
Most N2 NA sites associated with NAI resistance or (H)RI and/or contacting with the drug were under negative selection in both total and at least one of the different time periods analysed (temporal splitting) (Figure 6.9B). When not negatively selected, these sites were found to be experiencing an n.s. dN/dS<1. The positive selection detected at sites 148 and 151 contrasted with this overall scenario. As detailed above (see section 6.1.3.2.2), both sites were under PSP in the total time period and, specifically, since NAI introduction into clinic (1999 - 2008, and from 2009 onwards). Site 148 experienced a greater change, as was initially under n.s. dN/dS<1 (before 1999; Figure 6.9B).

Influenza B NA sites associated with NAI resistance or (H)RI and/or contacting with the drug were essentially under negative selection for B/VIC lineage and either under negative selection or experiencing an n.s. dN/dS<1 for B/YAM lineage, in the total time period analysed (Figure 6.9C). Site 395 was the only under PSP and in exclusively B/VIC-lineage, as detailed above in section 6.1.3.2.3. Few sites were found to be experiencing an n.s. dN/dS>1, including sites 71 and 149 in B/VIC-lineage NA, and sites 71, 395 and 449 in B/YAM-lineage NA. Only V71A and D149N amino acid substitutions known to confer RI were, however, among the polymorphisms identified (presented below in Figure 6.11C). Temporal splitting revealed an overall variation in the SP profile of the sites across the different time periods (Figure 6.9C), but with no similar or particular trend that could be explained by the underlying differences on NAI drug use. The only exception was site 395 that, although already experiencing an n.s. dN/dS>1 before 1999, started to be positively selected after the licensing of NAI drugs (see section 6.1.3.2.3 for detail). The higher number of conserved sites before 1999 for both influenza B lineages was most probably a result of the much smaller size of the sequence sub-dataset.

6.1.3.4 Overall Profiling of Site-Specific Selective Pressures in Human Influenza Virus Neuraminidase

The site-specific SP profiles obtained for the entire coding region of the different human influenza NA subtypes or lineages during the total time period (total datasets), were aligned using N2 codon coordinates (standard numbering scheme) (Figure 6.10). This not only allowed to have an overall picture of the site-specific SP acting on influenza NA but also to compare SP profiles of particular sites or regions across the different subtypes or lineages of human influenza NA.

Seven regions within the NA catalytic globular head domain were found to be comprised by 6 or more sites experiencing significant (negatively selected) or n.s. dN/dS<1 in all different virus subtypes or lineages. Specifically, regions (1) 87-92; (2) 164-171; (3) 177-186; (4) 227-233; (5) 254-261; (6) 275-284; and (7) 297-307 (N2 coordinates) (Figure 6.10). Considering that many of the amino acid substitutions at these sites are likely to be intolerable, particularly those at negatively selected sites [20], these regions may be suitable for drug targeting. In fact, region 227-233 (region 4) is partially included in the only universally conserved region among influenza A and B viruses (region 222-230). This conserved region has been pinpoint as a potential new target for NAI drugs [21]. The 150-cavity loop has also been considered as an attractive target for antiviral therapy [22] but all sites within this region except one (site 152) were found to be under positive selection or experiencing an n.s. dN/dS>1 for at least one virus subtype or lineage.



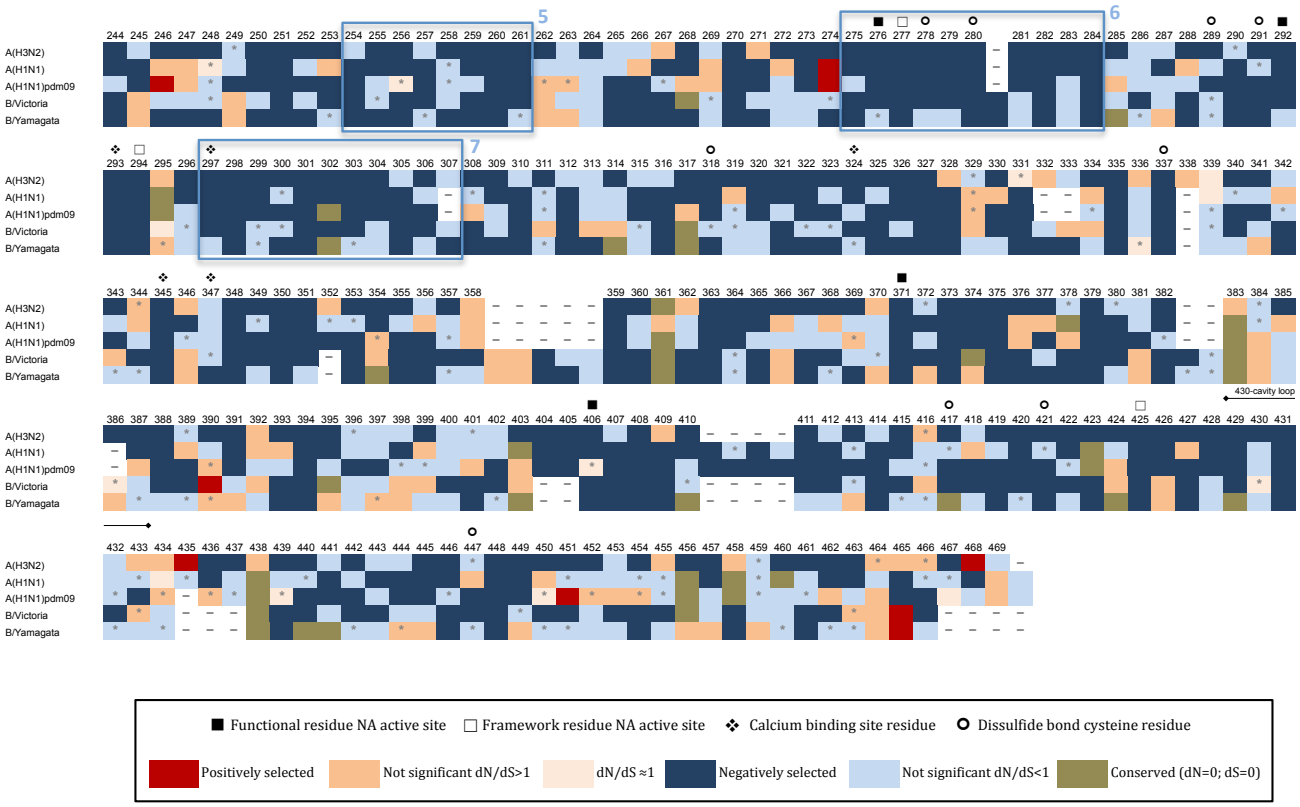


Figure 6.10 Overall mapping of site-specific selective pressure profiles across the different subtypes or lineages of human influenza virus neuraminidase.

The different human influenza virus neuraminidase (NA) site-specific selective pressure (SP) profiles were aligned using N2 codon coordinates (standard numbering scheme). The profiles were determined as described in Figure 6.9, using total NA sequence datasets (total time period). Similarly, individual results are indicated with an asterisk (*). Functional and framework NA active site residues were defined according to Colman *et al.* [19]; while calcium binding site and disulfide bound cysteine residues were based on Air [23], and on Colman and Ward [11] and Colman [9], respectively. The blue rectangles highlight the 7 regions that, based on their site-specific SP profiles ((not) significant dN/dS<1), may constitute potential new targets for NA inhibitor drugs.

6.1.4 Differential Analysis of Site-by-Site Selective Pressures Among Different Contexts of Neuraminidase Inhibitor Drug Use (temporal sub-datasets)

Differential site-by-site SP analysis allowed to identify which sites within the different human influenza NA subtypes or lineages experienced distinct SP among the different time periods analysed (temporal sub-datasets). Considering that the different time periods represented different contexts of global NAI drug use, it were only analysed in detail the differentially selected sites associated with NAI resistance or (H)RI and/or contacting directly or indirectly with the drug (NA active site). A list of all differentially selected sites can be found at Supplementary data, Tables S6.1 to S6.5.

Site 275 of seasonal N1 NA was found to be experiencing stronger SP (S_SP) in both 1999 to 2008 and from 2009 onwards time periods, compared to before 1999 (Table 6.4). This was in agreement with the variation observed in its SP profile across the different time periods, from n.s. dN/dS<1 to positively selected and then to n.s. dN/dS>1 (see Figure 6.9A). However, as above-mentioned, it is possible that differences in the strength of SP acting at this site may be just an artefact of the worldwide spread of a NA H275Y drug-resistant seasonal A(H1N1) variant since late 2007.

Table 6.4 Differentially selected sites in former seasonal A(H1N1), A(H3N2), B/Victoria and B/Yamagata-lineage virus neuraminidase associated with neuraminidase inhibitor resistance or (highly) reduced inhibition and/or contacting with the drug (active site).

Influenza virus subtype or lineage	Codon site	Site characteristics	Before 1999 (1) *vs* 1999-2008 (2)				1999-2008 (2) *vs* From 2009 onwards (3)				Before 1999 (1) *vs* From 2009 onwards (3)			
			p value	dN/dS_1	dN/dS_2	DIF	p value	dN/dS_2	dN/dS_3	DIF	p value	dN/dS_1	dN/dS_3	DIF
Former seasonal A(H1N1)	275	•••• ⓓ R OS; HRI OS+PER	0.00061	1.02E-09	12.65	***S_SP***	–	–	–	–	0.012	0.00	inf	***S_SP***
A(H3N2)	41	••• ↓ RI ZA	–	–	–	–	–	–	–	–	0.042	0.14	0.79	***S_SP***
	148	•••• ↓ E119V OS+ZA+PER+LAN	0.012	0.18	5.22	***S_SP***	–	–	–	–	0.0019	0.18	14.64	***S_SP***
B/Victoria	294	•••• □ ↓ (H)RI OS; RI PER	–	–	–	–	0.053	0.50	0.00	W_SP	–	–	–	–
B/Yamagata	154	•••• □	–	–	–	–	0.050	0.00	inf	***S_SP***	–	–	–	–
	197	•••• □ ↓ (H)RI OS+PER; RI ZA	–	–	–	–	0.050	inf	0.32	W_SP	–	–	–	–

vs: versus; dN: rate of non-synonymous substitutions; dS: rate of synonymous substitutions; DIF: Difference; R: Resistance; HRI: Highly reduced inhibition; RI: Reduced inhibition; (H)RI: HRI or RI; OS: Oseltamivir; ZA: Zanamivir; PER: Peramivir; LAN: Laninamivir; S_SP: Stronger Selective Pressure; W_SP: Weaker Selective Pressure; inf: infinite (dS=0)

Symbol legend:

••• Stalk domain •••• Catalytic globular head domain ⓓ Associated with NAI clinical resistance and (H)RI *in vitro*

↓ Associated with (H)RI by NAIs *in vitro* ↓ Associated with a synergistic effect on NAI (H)RI

Site-by-site selective pressures (SP) acting on influenza neuraminidase (NA) gene from two different time periods were compared in HyPhy, using the Muse-Gaut 94 model crossed with the model described as 012340 and previously determined by Akaike's Information Criterion as the best time reversible model for NA gene. The dash (-) represents no significant difference in the SP between that specific time periods, while significant differences involving stronger SP are highlighted in bold and italic. A list of all differentially selected sites within the different human influenza NA subtypes and lineages can be found at Supplementary data, Tables S6.1 to S6.4.

N2 NA site 148 was also found to be experiencing S_SP in both 1999 to 2008 and from 2009 onwards time periods, compared to before 1999. A S_SP was also detected at site 41 of N2 NA but only in the period of increased NAI drug use (from 2009 onwards) (Table 6.4). The significant differences at site 148 were in complete agreement with the variation previously observed in its SP profile, from n.s. dN/dS<1 before 1999 to positively selected in both 1999 to 2008 and from 2009 onwards time periods (see Figure 6.9B above).

Influenza B NA sites associated with NAI (H)RI and/or contacting with the drug were only differentially selected between 1999 to 2008 and from 2009 onwards, in both B/VIC and B/YAM lineages. Sites 294 and 197 in, respectively, B/VIC and B/YAM-lineage NA were found to be experiencing weaker SP (W_SP) in the period of increased NAI drug use (from 2009 onwards), while the converse was observed for site 154 in B/YAM-lineage NA (S_SP) (Table 6.4).

Regarding A(H1N1)pdm09 virus NA, sites 136, 247 and 295 were identified as differentially selected between the (pre-)pandemic and post-pandemic periods (Table 6.5). All three sites experienced S_SP in the latter period, during which NAI drug use was lower. Also, evidence for differential selection was considerably stronger for site 136 (lower p value). These significant differences were noted in the SP profile of the sites in each time period (see Figure 6.9A), except for site 295 that was continually under negative selection.

Table 6.5 Differentially selected sites in 2009 pandemic N1 NA associated with neuraminidase inhibitor resistance or (highly) reduced inhibition and/or contacting with the drug (active site).

Codon site	Site characteristics	p value	dN/dS		DIF
			(Pre-)Pandemic period	Post-pandemic period	
136 ••••	↓ HRI ZA+PER; RI LAN	0.00090	0.00	2.30	***S_SP***
247 ••••	↓ RI OS ↓ H275Y ZA+PER	0.047	2.26	inf	***S_SP***
295 •••• □	↓ HRI OS; RI PER	0.019	0.00	0.22	***S_SP***

dN: rate of non-synonymous substitutions; dS: rate of synonymous substitutions; DIF: Difference; HRI: Highly reduced inhibition; RI: Reduced inhibition; OS: Oseltamivir; ZA: Zanamivir; PER: Peramivir; LAN: Laninamivir; S_SP: Stronger Selective Pressure; W_SP: Weaker Selective Pressure; inf: infinite (dS=0)

Symbol legend:

•••• Catalytic globular head domain ↓ Associated with (H)RI by NAIs *in vitro* ↓ Associated with a synergistic effect on NAI (H)RI

Site-by-site selective pressures (SP) in the two different time periods were compared as described in Table 6.5. Significant differences involving stronger SP are highlighted in bold and italic. A list of all differentially selected sites within 2009 pandemic N1 NA is presented in Table S6.5, Supplementary data.

6.1.5 Frequency of amino acid substitutions conferring or enhancing neuraminidase inhibitor resistance or (highly) reduced inhibition in vitro in sequence databases (complementary study)

As an important complement to all data generated throughout this PhD study, total NA sequence datasets were further examined to determine the frequency of the amino acid

substitutions known to confer or enhance NAI resistance or (H)RI in each human influenza virus type or subtype (based on WHO [8] and WHO [16]). Total NA sequence datasets were obtained by merging the temporal sub-datasets constructed with all potentially complete NA coding sequences available at GISAID EpiFlu™ and NCBI Influenza Virus Resource databases (detailed in section 3.1.6, Material and Methods). The frequency at which the amino acid substitutions were detected is presented in Figure 6.11, separately by NA type and subtype (N1 (A); N2 (B); and influenza B (C)). Only the substitutions identified in at least one sequence are indicated.

Most amino acid substitutions conferring or enhancing NAI resistance or (H)RI in seasonal A(H1N1) subtype were identified at a frequency lower than 0.5% (0.1% to 0.4%; 1 to 7 sequences) (Figure 6.11A). The only exceptions were the N70S and H275Y substitutions that were present in, respectively, 27 (1.8%) and 585 (38.4%) sequences. The much higher frequency of H275Y substitution was already expected, giving the worldwide spread of an NA H275Y drug-resistant variant. NA D151G and I223V substitutions were identified together with H275Y in one sequence, constituting the only synergistic combinations identified. The synergistic I117V substitution and both E119V and R293K substitutions conferring (H)RI were not identified in any of the sequences.

The amino acid substitutions conferring or enhancing NAI resistance or (H)RI in current circulating A(H1N1) viruses (A(H1N1)pdm09) were essentially identified at a frequency of 0.1% or less (5 or less sequences) (Figure 6.11A). Exceptions include the synergistic D199N and S247N substitutions that were present in, respectively, 18 (0.5%) and 31 (0.9%) sequences; and the H275Y substitution that was found in 146 sequences (4.3%). However, neither D199N nor S247N, as well as none of the remaining synergistic substitutions, were identified together with the mutation conferring the (H)RI phenotype (H275Y; N295S+H275Y). Similarly, Q313K substitution was identified in 22 sequences (0.6%) but in none existed together with I427T (not detected; 0.0%), being the combination of both amino acid changes that is known to confer RI. In addition to I427T, the E119V/D and D199E substitutions conferring (H)RI were not detected in any of the sequences.

A - N1 NEURAMINIDASE

Codon site	Amino acid substitution	NAI susceptibility profile	FORMER SEASONAL A(H1N1) (N=1523; 1943-2010)	A(H1N1)pdm09 (N=3428; 2009-2013)
70	N70S	↓ RI ZA (N1)	1.8 (27)	-
117	I117M	↓ N295S+H275Y ZA (N1)	0.3 (5)	-
119	E119A	↓ RI OS+ZA+PER+LAN (N1pdm09)	-	0.03 (1)
	E119G	↓ HRI ZA+LAN; RI PER (N1pdm09) ↓ H275Y PER (N1pdm09)	-	0.03 (1)
136	Q136K	↓ (H)RI ZA+PER (N1+N1pdm09); RI LAN (N1pdm09)	0.4 (7)	0.03 (1)
	Q136R	↓ HRI ZA+PER; RI LAN (N1pdm09)	-	0.1 (5)
151	D151E	↓ RI ZA+PER (N1pdm09)	-	0.06 (2)
	D151N	↓ H275Y OS (N1)	0.3 (4)	-
	D151G	↓ H275Y OS+ZA+PER (N1)	0.1 (2)	-
155	Y155H	↓ (H)RI OS+ZA+PER (N1)	0.1 (1)	-
199	D199G	↓ RI OS (N1pdm09)	-	0.06 (2)
	D199N	↓ H275Y OS (N1pdm09)	-	0.5 (18)
223	I223K	↓ RI OS (N1pdm09) ↓ H275Y ZA (N1pdm09)	-	0.03 (1)
	I223R	↓ RI OS+ZA+PER (N1pdm09) ↓ H275Y ZA+PER (N1pdm09)	-	0.1 (5)
	I223V	↓ H275Y PER (N1+N1pdm09); N295S+H275Y OS+ZA (N1)	0.1 (2)	0.03 (1)
247	S247G	↓ RI OS (N1pdm09)	-	0.03 (1)
	S247N	↓ H275Y OS+ZA+PER (N1pdm09)	-	0.9 (31)
275	H275Y	ⓓ R OS; (H)RI OS+PER (N1+N1pdm09)	38.4 (585)	4.3 (146)
295	N295S	↓ (H)RI OS+PER (N1) / HRI OS; RI PER (N1pdm09)	0.1 (1)	0.03 (1)
313	Q313K [a]	↓ RI OS+ZA (N1pdm09)	-	0.6 (22) (0.0* (0))
325	N325K	↓ RI OS (N1pdm09)	-	0.03 (1)

B - N2 NEURAMINIDASE

Codon site	Amino acid substitution	NAI susceptibility profile	A(H3N2) (N=3712; 1968-2013)
41	E41G	↓ RI OS	0.05 (2)
119	E119D	↓ RI ZA	0.05 (2)
	E119V	↓ (H)RI OS	0.2 (8)
136	Q136K	↓ (H)RI ZA	0.2 (7)
148	T148I	↓ E119V OS+ZA+PER+LAN	2.7 (102)
151	D151E	↓ RI OS	0.8 (30)
	D151V	↓ HRI ZA	0.2 (8)
	D151A	↓ RI ZA	0.08 (3)
	D151G	↓ HRI ZA	1.9 (70)
222	I222T	↓ RI OS	0.1 (5)
226	Q226H	↓ RI OS	0.05 (2)
292	R292K	↓ (H)RI OS+ZA+PER	0.08 (3)

C - INFLUENZA B NEURAMINIDASE

Codon site	Amino acid substitution	NAI susceptibility profile	B/VICTORIA (N=1978; 1972-2013)	B/YAMAGATA (N=1441; 1973-2013)
71	V71A [a]	↓ RI PER+LAN	0.0 (0)	0.1 (2) (0.0* (0)
105	E105K	↓ RI OS+ZA+LAN; (H)RI PER	0.2 (4)	0.0 (0)
117	E117A	↓ HRI OS+ZA+PER+LAN	0.0 (0)	0.07 (1)
134	H134Y	↓ RI PER	0.05 (1)	0.0 (0)
138	Q138R	↓ RI ZA+LAN; (H)RI PER	0.05 (1)	0.0 (0)
	Q138K	↓ RI PER	0.05 (1)	0.0 (0)
139	P139S	↓ (H)RI OS+ZA+PER+LAN	0.05 (1)	0.0 (0)
140	G140R	↓ (H)RI OS+ZA+PER+LAN	0.05 (1)	0.0 (0)
142	Y142H	↓ RI PER	0.05 (1)	0.0 (0)
145	G145R [b]	↓ RI OS; HRI PER	0.2 (4) (0.05* (1))	0.0 (0)
148	G148R [c]	↓ RI PER	0.1 (2) (0.05* (1))	0.0 (0)
149	D149N	↓ RI PER	0.3 (5)	0.0 (0)
151	N151S	↓ RI ZA	0.0 (0)	0.07 (1)
197	D197E	↓ RI OS+ZA+PER	0.0 (0)	0.07 (1)
	D197N	↓ RI OS+ZA+PER	0.05 (1)	0.3 (4)
200	A200T	↓ RI OS+ZA	0.05 (1)	0.07 (1)
221	I221L	↓ HRI OS; RI ZA+PER+LAN	0.05 (1)	0.0 (0)
	I221T	↓ RI OS+ZA+PER	0.1 (2)	0.2 (3)
	I221V	↓ RI OS+PER	0.3 (6)	0.0 (0)
	I221N	↓ HRI OS+PER; RI ZA	0.05 (1)	0.07 (1)
245	A245T	↓ RI OS+ZA+PER	0.05 (1)	0.0 (0)
264	E264V	↓ RI PER	0.05 (1)	0.0 (0)
273	H273Y	↓ RI OS; (H)RI PER	0.0 (0)	0.07 (1)
294	N294S	↓ (H)RI OS; RI PER	0.05 (1)	0.0 (0)
343	K343E [c]	↓ RI PER	2.0 (39) (0.05* (1))	7.1 (102) (0.0* (0)
360	K360E	↓ HRI PER	0.1 (2)	0.0 (0)
374	R374K	↓ HRI OS+PER; (H)RI ZA	0.05 (1)	0.0 (0)
395	A395E	↓ RI OS+ZA	0.1 (2)	0.0 (0)
432	D432G	↓ RI PER	0.2 (3)	0.0 (0)
439	H439R [a]	↓ RI PER+LAN	0.1 (2) (0.0* (0))	0.0 (0)

Figure 6.11 Frequency of N1 (**A**), N2 (**B**) and influenza B (**C**) neuraminidase amino acid substitutions conferring or enhancing neuraminidase inhibitor resistance or (highly) reduced inhibition *in vitro* in sequence databases.

NAI: Neuraminidase inhibitor; RI: Reduced inhibition; HRI: Highly reduced inhibition; (H)RI: RI or HRI; OS: Oseltamivir; ZA: Zanamivir; PER: Peramivir; LAN: Laninamivir

Symbol legend:

ⓓ Associated with NAI clinical resistance and (H)RI *in vitro* ↓ Associated with (H)RI by NAIs *in vitro* ↓ Associated with a synergistic effect on NAI (H)RI

(Footnotes Figure 6.11 cont.)
The frequency of the amino acid substitutions was determined in total neuraminidase (NA) sequence datasets, which were obtained by merging the different temporal sequence datasets constructed with all potentially complete NA coding sequences available at GISAID EpiFlu™ and NCBI Influenza Virus Resource databases (see Material and Methods, section 3.1.6 for detail). The specific number of sequences containing the substitution is indicated under brackets after its frequency. Both total number of sequences enclosed in the dataset and time period covered are also indicated under brackets, below the designation of the human influenza virus subtype or lineage. NAI susceptibility profiles conferred by the amino acid substitutions were based on WHO [8] and WHO [16]. **Panel A**: [a] RI when combined with I427T substitution; **Panel C**: [a] RI when combined with each other; [b] (H)RI when combined with Y142H substitution; [c] RI when combined with each other. In the cases wherein is a combination of two amino acid substitutions that confers the (H)RI phenotype, it is first indicated the overall frequency at which the substitution was detected and only then, under brackets and highlighted with an asterisk (*), the frequency at which was identified with the other substitution. Amino acid substitutions are indicated using the influenza NA type or subtype-specific numbering scheme.

Regarding A(H3N2) subtype, most amino acid substitutions were present at frequencies that varied between 0.05% and 0.2% (2 to 8 sequences) (Figure 6.11B). NA D151E/G substitutions were exceptionally identified in 30 (0.8%) and 70 (1.9%) sequences, respectively. The synergistic T148I substitution was also identified in a higher number of sequences (102 sequences; 2.7%), but only in one existed together with the E119V substitution conferring (H)RI . Six amino acid substitutions known to confer (H)RI were not identify in any of the sequences - E119I, I222L, R224K, E276D, N294S, and R371K.

Influenza B NA amino acid substitutions conferring (H)RI were identified at very low frequencies that varied from 0.05% to 0.3% (1 to 6 sequences) for B/VIC and from 0.07% to 0.3% (1 to 4 sequences) for B/YAM lineage (Figure 6.11C). Additionally, many of the substitutions were only present in B/VIC-lineage virus sequences. The K343E substitution was identified at a higher frequency in both lineages (2.0% B/VIC; 7.1% B/YAM), but only in a single B/VIC-lineage virus sequence (0.05%) was detected together with G148R, resulting in the combination that is know to confer RI. Several amino acid substitutions known to confer (H)RI were not detected in any of the sequences from both lineages, including I114R, E117D/G/V, R150K, D197Y, R292K, G407S, S440L, and M449V substitutions.

6.2 DISCUSSION

Global and site-specific SP acting on influenza NA were for the first time estimated for all virus subtypes and lineages circulating among humans. Moreover, it was not only

considered a total time period but also different sub-time periods defined by differences on NAI global use. Total NA sequence datasets enclosed a very large number of sequences of worldwide circulating viruses that exceeded 3400 sequences for A(H3N2) and A(H1N1)pdm09 subtypes and varied between approximately 1450 and 2000 sequences for seasonal A(H1N1) and influenza B/VIC and B/YAM subtype/lineages. These differences were in agreement with the greater incidence rates and higher frequency of A(H3N2) seasonal epidemics, comparing to A(H1N1) and type B epidemics, and the pandemic nature of A(H1N1)pdm09 viruses [24,25]. All sequence datasets with exception of A(H1N1)pdm09 dataset also covered an extensive time period that varied from 41 to 68 years, according to the virus subtype or lineage. Such large number of sequences and extensive time period were only analysed in a previous study conducted with N1 NA sequences of A(H1N1) viruses from different hosts and for exclusively seasonal human influenza A(H1N1) subtype (1735 sequences; until 2010) [26]. Temporal NA sequence sub-datasets varied widely in their size and/or time period covered. As expected, the first temporal sub-dataset (before 1999) contained a much smaller number of sequences for all virus subtypes or lineages (not applied to A(H1N1)pdm09). Both polymerase chain reaction (PCR) and automated Sanger sequencing technologies were only developed in the 1980s [27,28], meaning that all sequences from previously circulating viruses were obtained through specific retrospective studies. The larger size of A(H3N2) and, particularly, influenza B/VIC and B/YAM-lineage from 2009 onwards sub-datasets, compared to 1999 to 2008 sub-datasets, may be related with the efforts that have been made since the 2009 A(H1N1) pandemic to improve global influenza surveillance [29]. Although not uniformly distributed, sequences from all years or epidemiological weeks (A(H1N1)pdm09) within the time period covered and from all 5 worldwide continents were present in the different (sub-)datasets. Only for seasonal A(H1N1) and influenza B/VIC and B/YAM-lineage before 1999 sub-datasets this was not observed. A more detailed geographic analysis showed also that most (sub-)datasets included sequences from all 18 influenza transmission zones established by WHO. Genetic diversity of total sequence datasets ordered as follows: A(H3N2) > seasonal A(H1N1) > B/Victoria ≈ B/Yamagata > A(H1N1)pdm09. This was in agreement with the differences in the evolutionary antigenic drift of the different human influenza NA types or subtypes. N2 NA gene is known to evolve rapidly as a single lineage, followed by seasonal N1 NA gene that, although mutating at a similar rate, has been experiencing weaker antigenic drift [30]. Influenza B NA gene usually evolves at a two to three times lower rate than N2 and

seasonal N1 NA genes [30]; while 2009 pandemic N1 NA gene has been evolving at a very slow rate, mainly as a result of a relatively low immunological pressure (new virus to which many people had no pre-existing immunity) [31]. The decreasing nucleotide diversity of seasonal A(H1N1), A(H3N2) and, in a lesser extent, B/VIC-lineage temporal sub-datasets was probably an artefact of their different size and/or time length. The approximate two-fold increase in the genetic diversity of A(H1N1)pdm09 temporal sub-datasets may be associated with an increase in the human population immunity against the virus, probably as a result of infection or vaccination.

6.2.1 Global Selective Pressure on Human Influenza Virus Neuraminidase Gene

Global dN/dS ratios for influenza NA gene were similar and less than 1 not only across the different virus subtypes or lineages in total time period but also across the different time periods in each virus subtype or lineage. This indicated that NSP forces of similar magnitude have been acting on the NA gene of all human influenza viruses, and that neither the introduction of NAIs into clinic and/or its increased use during 2009 A(H1N1) pandemic had an impact on the overall strength of NSP. Global dN/dS ratio estimates in total time period (0.21 - 0.26) were similar to those obtained in previous studies (0.19 - 0.30) [20,26,32-39].

6.2.2 Site-by-Site Selective Pressures on Human Influenza Virus Neuraminidase Gene

Most variable sites within the NA gene of all human influenza virus subtypes or lineages were found to be experiencing a dN/dS<1 that was statistically significant for approximately or slightly more than half of them in total time period - 41.6% to 52.3% of all variable sites under negative selection. Only in A(H3N2) virus NA gene this percentage was even higher, reaching 67.3%. The high percentage of negatively selected sites in all virus subtypes or lineages may be a result of the intensive sequence sampling that may have allowed for sequences representing transitory sub-optimal viral sub-populations to be present. Nevertheless, it was in agreement with the significant dN/dS<1 value obtained for the entire gene (global dN/dS ratio). Very few NA sites were under positive

selection, varying from 1 to 8 (0.2% to 1.8% of all variable sites) in total time period, according to the virus subtype or lineage.

The differences in the distribution of the variable sites across the different SP profiles over the different time periods were probably just artefacts of the different size of the sequence sub-datasets.

6.2.2.1 Positively Selected Sites

Site 275 was the only positively selected site in seasonal influenza N1 NA in either the total time period or the different time periods analysed (only between 1999 and 2008). Moreover, the H275Y substitution known to confer clinical resistance to OS and (H)RI by OS and PER *in vitro* was the only amino acid polymorphism detected at this site, evidencing the occurrence of directional PSP. In fact, from 2009 onwards the site was no longer under PSP and H275Y was already fixed in the virus population (96.4% H275Y). Previous studies have also identified site 275 as the only [40,41] or as one of the positively selected sites [26] in seasonal N1 NA, with two of them suggesting a significant role for positive selection in the rapid and global spread of a NA H275Y OS-resistant variant since late 2007 [26,40]. Further investigation of the PSP acting on site 275 between 1999 and 2008 did not, however, support that. The site was found under PSP from 1999 to 2007 but not from 1999 to 2006, indicating that positive selection may have only started in 2007. Based on this, it may be just an artefact of the global spread of the drug-resistant variant that may otherwise have resulted from genetic hitchhiking to advantageous mutations located in any other segment of viral genome, as has been suggested in several studies [42-44]. This can be the case of PB2 P453S and/or PB1 N642S amino acid substitutions identified in this PhD study as specific of NA H275Y OS-resistant seasonal A(H1N1) viruses (see Chapter 5 for detail), but which effect on virus fitness is still unknown. It will be interesting to investigate if positive selection has been acting on theses sites and if so, if it has favoured those particular amino acid changes.

Sites 44, 148, 151, 435 and 468 in human influenza N2 NA were found to be evolving under diversifying PSP in total time period. PSP revealed to be particularly strong at sites 148 and 151 associated with (H)RI by NAI drugs and located closely to the NA active site. In fact, these were the only that were already identified under PSP, although in just one

previous study [38]. All other previous studies detected a completely divergent set of sites under PSP, most probably due to the much smaller size of sequence datasets (284 and 345 sequences) [20,36,37]. Both sites 148 and 151 have, however, shown to be implicated in cell culture selection. Several amino acid changes at these sites, including the (H)RI-conferring D151A/G substitutions and the synergistic T148I substitution, have only been detected in cell-grown virus isolates and not in clinical specimens [45-47]. Considering that most influenza gene sequences available at public databases were obtained from virus isolates, it is possible that the strong PSP detected at both sites may be just an artefact from cell culture. Based on this, no inference can be made about the fact that both sites were only under PSP in the period of use (from 1999 to 2008) and increased use (from 2009 onwards) of NAI drugs, and about the increase in the strength of PSP acting at site 148 between the two time periods. Positively selected sites 435 and 468 may constitute key immune epitope contacting residues, as evidenced by their location at the surface of the protein. Antibody epitopes on human influenza N2 NA are still very poorly characterized (only Mem5 epitope is completely mapped to date [17]) and thereby there is still much information missing.

Influenza B/VIC-lineage NA presented the highest number of sites under PSP, which was unexpected considering that influenza B NA evolves at a two to three times lower rate than seasonal N1 and N2 NA [30]. A total of 8 sites were found to be under PSP in total time period, half of them located in the stalk and the other half in the catalytic globular head but apart from the active site. Among these latter, it was site 395 associated with RI by OS and PER *in vitro*. All positively selected sites except one (site 73) were found to be under diversifying PSP. Considering that the RI-conferring A395E amino acid substitution was one of the polymorphisms detected at site 395, a variant carrying such amino acid change can be at potential risk of spreading. However, latest global susceptibility surveillance data (2014/2015 season) showed still no evidence of that [48]. Site 395 was the only to be continually under PSP after the licencing of NAI drugs into the clinic (from 1999 to 2008, and from 2009 onwards). Although this seemed to indicate a potential role of drug-selective pressure, it is more likely that the site was under continuous SP from the host´s immune system, based on its location at the surface of the protein. According to its structural location, positively selected site 465 may also constitute a potential epitope contacting residue. The identification of potential antibody contacting residues in influenza B NA is particularly relevant as no epitope was completely mapped to date.

Only few amino acid residues that changed in antibody-selected escape mutants are known [49].

All positively selected sites in B/YAM-lineage NA in total time period appeared to be evolving under directional PSP, in spite of the diversity of amino acid polymorphisms identified in most of them. Sites 65, 73 and 465 were also under PSP in B/VIC lineage, which for site 465 supported its potential role as epitope contacting residue. Site 65 was detected under PSP in a previous study but only for B/YAM lineage [38]. Sites 42, 106 and 198 constituted the remaining sites under PSP. Due to its location at the surface of the protein, site 106 may also constitute a potential epitope contacting residue. Site 198 near the enzymatic active site was the most strongly selected site. Drug-selective pressure might have been in the origin of this stronger PSP, as evidenced by the detection of PSP in only the periods of use (from 1999 to 2008) and increased use (from 2009 onwards) of NAI drugs. However, there was no consensus from the two methods used for the variation in the strength of PSP acting on this site between the two time periods. Site 198 is not associated with (H)RI by NAI drugs but lies adjacently to site 197 in which have been detected several amino acid substitutions conferring (H)RI by all NAIs except LAN (D197E/N/Y) [8].

Positively selected sites in 2009 pandemic N1 NA in total time period included sites 46 and 74 within the stalk domain; sites 95 and 450 under selection of the host´s immune system (established epitope contacting residues); and sites 247 and 275 located closely to the active site and associated with NAI resistance and/or (H)RI. All sites were found to be evolving under diversifying PSP, except site 275 in which was only detected the H275Y amino acid variant known to confer clinical resistance to OS and (H)RI by OS and PER *in vitro*. Site 275 was also found to be experiencing stronger PSP in the (pre-) pandemic period, during which NAI drugs, particularly OS, were more widely used. However, this may be just a sampling artefact as most NA H275Y OS-resistant viruses identified in this period were recovered from patients undergoing OS treatment [50] and, due the lack of information on antiviral therapy in sequence databases, sequences from treated patients could not be excluded from the study sample. The continuity of positive selection throughout the post-pandemic period suggested that PSP may have been playing an important role in the low-level and locally variable spread of NA H275Y A(H1N1)pdm09 viruses that has been observed since 2011, among untreated community

patients with either or not an epidemiological link (cluster) [51-53]. Positive diversifying selection at site 247 may also constitute a threat to NAI use as both RI-conferring S247G and synergistic S247N amino acid substitutions were among the polymorphisms identified. Variants carrying such amino acid changes can be therefore at potential risk of spreading, although latest global susceptibility surveillance data (2014/2015 season) showed still no evidence of that [48]. Positive selection at site 46 may be in part related with the gain of a N-linked glycosylation site - I46T amino acid substitution (Asparagine (N) at position 44). N-linked glycosylation is known to be an important evolutionary mechanism of influenza viruses and the increase in the number of glycosylation sites showed to be characteristic of the early stages of evolution [54]. The fact that the site was only positively selected during the (pre-)pandemic period supports this hypothesis.

6.2.2.2 Selective Pressure on the Sites Associated with Resistance or (Highly) Reduced Inhibition by Neuraminidase Inhibitors and/or Contacting with the Drug

NA sites associated with NAI resistance or (H)RI and/or contacting with the drug were essentially under negative selection (A(H3N2), B/VIC-lineage) or either under negative selection or experiencing a n.s. dN/dS<1 (seasonal and 2009 pandemic A(H1N1), B/YAM-lineage) in total time period. This can be explained by the key functional role that most of these sites have on the sialidase activity of influenza NA protein, considering their position at or near the enzymatic active site (drug-binding pocket). Moreover, Lentz and colleagues showed that site-directed mutagenesis of active site residues result mainly in a complete loss of enzymatic activity [55], evidencing that amino acid changes at these sites are unlikely to be advantageous.

Positive selection at site 275 in seasonal N1 NA and sites 148 and 151 in N2 NA may be simply an artefact from, respectively, the global spread of a drug-resistant variant and cell culture, as above-mentioned. The same does not apply to the positive selection at site 395 in B/VIC-lineage NA and sites 275 and 247 in 2009 pandemic N1 NA that is otherwise concerning. NA A395E (influenza B), H275Y and S247G/N (A(H1N1)pdm09) amino acid substitutions known to confer or enhance NAI resistance or (H)RI were the single or one of the polymorphisms identified at these sites, meaning that variants carrying such amino acid changes can be at potential risk of spreading. In fact, NA H275Y

drug-resistant A(H1N1)pdm09 viruses have already spread in the community, but only locally and at low-level, as above-mentioned.
Site 199 in 2009 pandemic N1 NA and sites 71 and 149 in, respectively, both B lineage or only B/VIC-lineage NA, should also be closely monitored as were found to be experiencing a n.s. dN/dS>1 and the amino acid variant(s) conferring and/or enhancing (H)RI was/were among the polymorphisms identified.

The SP profile of the sites associated with resistance or (H)RI and/or contacting with the drug either not varied across the different time periods (A(H1N1)pdm09, A(H3N2)) or varied, but with no similar or particular trend that could be explained by the underlying differences in NAI drug use (seasonal A(H1N1), influenza B/VIC and B/YAM lineages). In fact, it is possible that some of the variations observed were just related with the different size of sequence sub-datasets. It can be therefore assumed that neither the introduction of NAI drugs into clinic nor its increased use during 2009 A(H1N1) pandemic had an apparent impact on the SP forces acting on these particular sites. Sites 148 and 151 in N2 NA and site 395 in B/VIC-lineage NA were continually under PSP after NAI introduction into clinic but, as above-mentioned, positive selection may be just an artefact from cell culture (sites 148 and 151) or a result of the selection from the host´s immune system (site 395, potential epitope contacting residue) and not drug use. Also, only the differences in the SP on site 148 were found to be statistically significant (differential SP analysis). Evidence for significant differences between the different time periods was also obtained for site 275 in seasonal N1 NA that was under significant and n.s. dN/dS>1 after the licensing of NAI drugs, and for few other sites with a less marked variation in their SP profile. Specifically, site 41 in N2 NA, site 294 in B/VIC-lineage NA, sites 154 and 197 in B/YAM-lineage NA, and sites 136, 247 and 295 in 2009 pandemic N1 NA. The significant S_SP acting on site 154 of B/YAM-lineage NA from 2009 onwards may in fact be associated with drug-selective pressure. Not only the increase in the strength of SP was in agreement with the increase in NAI drug use, but also site 154 is a framework residue of the active site, contacting indirectly with the drug. The significant S_SP acting on seasonal N1 NA site 275 in both from 1999 to 2008 and from 2009 onwards periods may otherwise be just an artefact from the global spread of a NA H275Y drug-resistant variant, as above-mentioned, while the position of site 41 in the stalk domain of N2 NA makes it unlikely to be affected by drug-selective pressure. All

remaining significant differences in SP were not in agreement with the differences in NAI drug use.

6.2.2.3 Overall Site-Specific Selective Pressures in Human Influenza Virus Neuraminidase

Seven regions within the catalytic globular head domain were found to be experiencing significant or n.s. dN/dS<1 in all different subtypes or lineages of human influenza NA, showing to be suitable for drug targeting. Specifically, regions (1) 87-92; (2) 164-171; (3) 177-186; (4) 227-233; (5) 254-261; (6) 275-284; and (7) 297-307 (N2 coordinates). Region 227-233 (region 4) is partially included in the only universally conserved region among influenza A and B viruses (region 222-230). Due to its highly conserved structure, this region has been continuously considered as an attractive new drug target and the recent study of Doyle *et al.* [21] provided the functional evidence for that, showing its critical role in NA enzymatic activity and viral replication. No information was found about any of the other regions here identified. The 150-cavity loop has also been proposed as potential new target but, considering that all sites within this region except one were experiencing significant or n.s. dN/dS>1 in at least one NA subtype or lineage, this may not be a suitable choice.

6.2.3 Frequency of amino acid substitutions conferring or enhancing neuraminidase inhibitor resistance or (highly) reduced inhibition in vitro in sequence databases

Most amino acid substitutions known to confer or enhance NAI resistance or (H)RI in the different human influenza virus types or subtypes were identified at very low frequencies (<0.5%) in public influenza sequence databases. These very low frequencies were already expected since all amino acid substitutions except N1 NA H275Y have been only rarely reported [56]. In this study, H275Y amino acid substitution was detected in approximately 40% of the seasonal A(H1N1) virus sequences, with nearly all belonging to the period in which circulated the fit and transmissible NA H275Y drug-resistant variant that spread globally. It was also detected in more than 4% of the A(H1N1)pdm09 virus sequences, occurring at an approximately two-fold higher frequency than that reported through antiviral susceptibility monitoring activities (<2%)[57]. This difference

can be related with the extremely high number of sequences analysed (3428 sequences) that most probably included sequences from laboratories that are not generating and reporting antiviral susceptibility data. Three synergistic amino acid substitutions - D199N and S247N (A(H1N1)pdm09) and T148I (A(H3N2)), were identified at slightly higher frequencies than the majority of the amino acid changes at that particular subtype, but only T148I was identified together with the substitution conferring the (H)RI phenotype and in only one sequence. The higher frequency at which D151E/G substitutions were identified in A(H3N2) subtype (0.8% and 1.9%, respectively) was probably related with the well-known association of the site to cell culture selection. Seasonal N1 NA N70S substitution was also identified at a slightly higher frequency (1.8%), but nearly all sequences harbouring this amino acid change were from before 1999. This is a substitution that only recently was found to affect the *in vitro* susceptibility to ZA [58]. Most of the detected influenza B NA amino acid changes conferring (H)RI were exclusive of B/VIC lineage. The frequency of (H)RI-conferring NA substitutions has already showed to differ substantially between the two influenza B lineages in a recent study [59], but this preference for B/VIC lineage was not noted. However, only 7 positions in influenza B NA were previously analysed, contrasting with the 31 positions now studied. The single occurrence of most amino acid changes in B/VIC-lineage viruses can mean that these viruses have a higher capacity to tolerate (H)RI-conferring NA substitutions, compared to B/YAM-lineage viruses.

6.3 CONCLUSIONS

This is one of the most comprehensive studies on the SP acting on influenza NA performed to date, involving the analysis of an extremely large number of NA gene sequences that, although not equally distributed over time and geographic regions, covered extensive time periods and all 5 worldwide continents and 18 WHO influenza transmission zones. It also involved the analysis of all different subtypes or lineages of human influenza NA that were for the first time studied together, under the same approaches and methodologies. A temporal splitting approach based on differences in NAI drug use was also used for the first time, allowing to infer about the impact of NAI introduction into clinic and/or its increased use during 2009 A(H1N1) pandemic on the global and site-specific SP acting on influenza NA (drug target). Potential interferences of

sampling size and cell culture implied, however, a careful interpretation of some results. Overall, this study contributed at a better understanding of the SP forces acting on the NA gene of all different subtypes or lineages of human influenza virus and of the role of PSP in the evolutionary pathways to NAI resistance or (H)RI. It also contributed at identifying potential epitope contacting residues, which is an area that is still poorly studied for NA; and at finding potential new druggable targets, which is now a priority in influenza given the limited repertoire of effective antivirals available. In a broader-sense, it also contributed at a better understanding of the evolutionary dynamics of influenza NA.

BOX 6.2 - MAIN FINDINGS

- NSP forces of similar magnitude have been acting on the NA gene of all human influenza virus subtypes or lineages (overall strength of selection).
- Similar distribution of site-specific SP profiles across the different subtypes or lineages of human influenza NA, with a high percentage of sites under negative selection (41.6% to 67.3%).
- Few positively selected sites (1 to 8 sites, according to the virus subtype or lineage), most lying at the catalytic globular head domain.
- Influenza B/VIC-lineage NA contained the highest number of sites under PSP (8 sites)
- Sites 435 and 468 in N2 NA, 395 and 465 in B/VIC-lineage NA, 106 and 465 in B/YAM-lineage NA, and 95 and 450 in 2009 pandemic N1 NA may be under positive selection from the host´s immune system - potential epitope contacting residues.
- Identification of 7 potential new regions for drug targeting.

Specific objective 3(a)

- NA sites associated with NAI resistance or (H)RI and/or contacting with the drug were essentially under negative selection (A(H3N2), B/VIC-lineage) or either under negative selection or experiencing n.s. dN/dS<1 (seasonal and 2009 pandemic A(H1N1), B/YAM-lineage).
- Positive selection at site 275 in seasonal N1 NA and sites 148 and 151 in N2 NA may be simply artefacts from, respectively, the global spread of a NA H275Y drug-resistant variant and virus isolation and propagation in cell culture.
- Positive directional selection at site 275 in 2009 pandemic N1 NA may be playing a role in the low-level and locally variable spread of NA H275Y drug-resistant A(H1N1)pdm09 viruses that has been observed among untreated community patients.
- Positive diversifying selection at site 395 in B/VIC-lineage NA and site 247 in 2009 pandemic N1 NA may lead to the emergence and spread of RI A395E variant and both synergistic HRI H275Y/S247N and RI S247G variants.
- Emergence of amino acid variants at site 199 in 2009 pandemic N1 NA and sites 71 and 149 in, respectively, both influenza B lineage NA and only B/VIC-lineage NA should be closely monitored (n.s. dN/dS>1).

BOX 6.2 - MAIN FINDINGS (cont.)

Specific objective 3(b)

- NAI introduction into clinic and/or its increased use during 2009 A(H1N1) pandemic had apparently no impact on the global and site-specific SP acting on the different subtypes or lineages of human influenza virus NA.
- Framework active site residue 154 of B/YAM-lineage NA may constitute the only exception, evolving under significant S_SP after the increased use of NAI drugs in the pandemic period.

Complementary study on the frequency of amino acid substitutions conferring or enhancing NAI resistance or (H)RI in vitro

- Amino acid substitutions known to confer or enhance NAI resistance or (H)RI in the different human influenza virus types or subtypes were mainly identified at very low frequencies (<0.5%) in public influenza sequence databases.
- N1 H275Y amino acid substitution identified in ≈40% of seasonal A(H1N1) virus sequences (global spread of drug-resistant variant) and in 4% of A(H1N1)pdm09 virus sequences.
- Higher frequency of D151E/G amino acid substitutions among A(H3N2) virus sequences probably associated with the implication of the site in cell culture selection.
- Most detected influenza B NA amino acid changes conferring (H)RI were exclusive of B/VIC lineage, suggesting that B/VIC-lineage viruses may have a higher capacity to tolerate these particular amino acid changes, compared to B/YAM-lineage viruses.

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SUPPLEMENTARY DATA

Table S6.1 Former seasonal N1 neuraminidase sites identified as differentially selected between the different time periods analysed (temporal sub-datasets).

Codon site	Site characteristics	Before 1999 (1) *vs* 1999-2008 (2)				1999-2008 (2) *vs* 2009 onwards (3)				Before 1999 (1) *vs* 2009 onwards (3)			
		p value	dN/dS_1	dN/dS_2	DIF	p value	dN/dS_2	dN/dS_3	DIF	p value	dN/dS_1	dN/dS_3	DIF
5	•	0.045	8.51E-10	1.48	***S_SP***	–	–	–	–	–	–	–	–
12	••	0.026	0.00	1.24	***S_SP***	–	–	–	–	–	–	–	–
16	••	0.021	1222104922.51	0.43	W_SP	–	–	–	–	–	–	–	–
17	••	–	–	–	–	0.055	0.00	0.64	***S_SP***	–	–	–	–
20	••	–	–	–	–	0.017	1.36	0.11	W_SP	–	–	–	–
37	•••	–	–	–	–	0.0059	inf	0.00	W_SP	0.049	inf	4.37E-16	W_SP
48	•••	0.019	0.078	713677715.96	***S_SP***	–	–	–	–	–	–	–	–
62	•••	0.0039	0.00	2.36	***S_SP***	–	–	–	–	–	–	–	–
67	•••	–	–	–	–	–	–	–	–	0.0072	0.00	inf	***S_SP***
80	••••	–	–	–	–	0.025	inf	0.00	W_SP	–	–	–	–
89	••••	0.048	inf	3.49E-09	W_SP	–	–	–	–	–	–	–	–
91	••••	–	–	–	–	0.019	0.00	inf	***S_SP***	–	–	–	–
122	••••	0.050	4.76E-10	77834796.33	***S_SP***	0.053	inf	0.00	W_SP	–	–	–	–
125	••••	–	–	–	–	0.033	0.00	inf	***S_SP***	–	–	–	–
126	••••	–	–	–	–	0.036	0.43	0.00	W_SP	–	–	–	–
131	••••	0.025	185788582.22	3.56E-09	W_SP	–	–	–	–	0.049	inf	0.00	W_SP
157	••••	0.00020	1066150198.03	3.48E-09	W_SP	–	–	–	–	–	–	–	–
168	••••	–	–	–	–	0.036	9.70E-16	0.50	***S_SP***	–	–	–	–
177	••••	–	–	–	–	–	–	–	–	–	–	–	–
214	••••	–	–	–	–	–	–	–	–	0.049	0.00	1.62	***S_SP***
222	•••• ↓	0.047	1339322283.05	0.81	W_SP	–	–	–	–	–	–	–	–
232	••••	–	–	–	–	–	–	–	–	0.047	inf	0.00	W_SP
236	••••	0.038	0.40	8.36E-10	W_SP	0.0071	0.00	inf	***S_SP***	–	–	–	–
248	•••• ◆	–	–	–	–	–	–	–	–	0.053	3.90	0.21	W_SP
250	•••• ◆	–	–	–	–	0.023	0.46	2.03	***S_SP***	–	–	–	–
253	••••	0.035	inf	4.17E-09	W_SP	–	–	–	–	–	–	–	–
263	••••	0.050	551573989.33	0.38	W_SP	–	–	–	–	–	–	–	–
264	••••	–	–	–	–	0.027	0.32	inf	***S_SP***	–	–	–	–
266	••••	–	–	–	–	0.018	inf	0.00	W_SP	–	–	–	–
275	•••• ⊕	0.00061	1.02E-09	12.65	***S_SP***	–	–	–	–	0.012	0.00	inf	***S_SP***
284	••••	–	–	–	–	–	–	–	–	–	–	–	–
325	••••	–	–	–	–	–	–	–	–	0.043	0.00	inf	***S_SP***
327	••••	0.0043	376985575.57	2.08E-09	W_SP	–	–	–	–	–	–	–	–
332	••••	0.0058	0.078	2.24	***S_SP***	–	–	–	–	–	–	–	–
336	••••	–	–	–	–	–	–	–	–	0.022	inf	0.00	W_SP
338	•••• ◆	0.049	1.12E-09	0.80	***S_SP***	–	–	–	–	–	–	–	–
339	•••• ◆	–	–	–	–	0.046	0.48	inf	***S_SP***	–	–	–	–
341	•••• ◆	0.030	0.00	inf	***S_SP***	–	–	–	–	0.022	0.00	inf	***S_SP***
346	••••	–	–	–	–	–	–	–	–	0.031	inf	0.072	W_SP
354	••••	0.031	1.16E-09	1.30	***S_SP***	–	–	–	–	0.015	0.00	inf	***S_SP***
377	••••	–	–	–	–	0.016	0.00	inf	***S_SP***	–	–	–	–
385	••••	–	–	–	–	–	–	–	–	0.037	inf	0.00	W_SP
389	••••	0.035	857789580.30	0.54	W_SP	0.041	0.54	inf	***S_SP***	–	–	–	–
416	••••	–	–	–	–	–	–	–	–	0.048	0.00	inf	***S_SP***
419	••••	–	–	–	–	–	–	–	–	0.014	0.00	inf	***S_SP***
424	••••	–	–	–	–	0.049	0.00	inf	***S_SP***	0.046	0.00	inf	***S_SP***
437	••••	–	–	–	–	0.047	0.27	inf	***S_SP***	–	–	–	–
449	••••	0.042	5.74E-10	0.36	***S_SP***	–	–	–	–	–	–	–	–
451	••••	–	–	–	–	–	–	–	–	0.039	0.16	inf	***S_SP***
452	••••	–	–	–	–	0.010	0.90	0.00	W_SP	–	–	–	–

Dif: Difference; dN: rate of non-synonymous substitutions; dS: rate of synonymous substitutions; (H)RI: (Highly) reduced inhibition; inf: infinite (dS=0); NAI: Neuraminidase inhibitor; S_SP: Stronger Selective Pressure; vs: versus; W_SP: Weaker Selective Pressure

Symbol legend:

• N-terminal cytoplasmic domain •• Transmembrane domain ••• Stalk domain •••• Catalytic globular head domain

◆ Potential epitope contacting residue (essential for antibody binding) ⊕ Associated with NAI clinical resistance and (H)RI in vitro

↓ Associated with a compensatory fitness effect on NAI resistant or (H)RI variants

Site-by-site selective pressures (SP) between two different time periods were compared as described in Table 6.5. The dash (-) represents no significant difference, while significant differences involving stronger SP are highlighted in bold and italic.

Table S6.2 N2 neuraminidase sites identified as differentially selected between the different time periods analysed (temporal sub-datasets).

Codon site	Site characteristics	Before 1999 (1) *vs* 1999-2008 (2)				1999-2008 (2) *vs* 2009 onwards (3)				Before 1999 (1) *vs* 2009 onwards (3)			
		p value	dN/dS_1	dN/dS_2	DIF	p value	dN/dS_2	dN/dS_3	DIF	p value	dN/dS_1	dN/dS_3	DIF
2	•	–	–	–	–	0.00015	2.62	0.046	W_SP	–	–	–	–
10	••	–	–	–	–	0.026	1.84	0.086	W_SP	–	–	–	–
11	••	–	–	–	–	0.054	0.15	1.1E-15	W_SP	–	–	–	–
14	••	0.00026	inf	0.046	W_SP	–	–	–	–	0.00026	inf	0.00	W_SP
17	••	–	–	–	–	–	–	–	–	0.010	inf	0.29	W_SP
25	••	0.027	0.00	inf	***S_SP***	–	–	–	–	–	–	–	–
29	••	–	–	–	–	–	–	–	–	0.0054	0.59	1.7E-07	W_SP
31	••	–	–	–	–	–	–	–	–	0.045	0.37	0.061	W_SP
39	•••	0.016	0.61	0.00	W_SP	–	–	–	–	–	–	–	–
41	••• ↓	–	–	–	–	–	–	–	–	0.042	0.14	0.79	***S_SP***
55	•••	–	–	–	–	0.0092	1.04	0.00	W_SP	0.020	inf	0.00	W_SP
60	•••	–	–	–	–	0.047	0.16	0.66	***S_SP***	0.046	0.00	0.66	***S_SP***
66	•••	–	–	–	–	0.036	0.27	0.00	W_SP	–	–	–	–
69	•••	0.020	0.53	inf	***S_SP***	–	–	–	–	–	–	–	–
74	•••	–	–	–	–	0.0039	0.042	0.89	***S_SP***	0.0095	0.052	0.89	***S_SP***
76	••••	0.037	0.00	0.15	***S_SP***	–	–	–	–	0.045	0.00	0.13	***S_SP***
78	••••	–	–	–	–	0.045	0.31	0.00	W_SP	–	–	–	–
94	••••	–	–	–	–	–	–	–	–	0.047	0.42	0.00	W_SP
120	••••	0.032	9.02	1.19	W_SP	0.0012	1.19	3.06E-15	W_SP	9.75E-06	9.02	0.00	W_SP
126	••••	–	–	–	–	0.00071	inf	0.24	W_SP	0.0035	inf	0.24	W_SP
141	••••	0.0025	3.17	6.37E-16	W_SP	0.0010	0.00	4.38	***S_SP***	–	–	–	–
147	•••• ◆	0.031	0.49	9.44	***S_SP***	0.0039	9.44	0.55	W_SP	–	–	–	–
148	•••• ↓	0.012	0.18	5.22	***S_SP***	–	–	–	–	0.0019	0.18	14.64	***S_SP***
150	•••• ◆	0.035	0.19	4.48	***S_SP***	–	–	–	–	0.025	0.19	5.38	***S_SP***
153	•••• ◆	0.024	inf	0.20	W_SP	–	–	–	–	7.63E-05	inf	3.7E-16	W_SP
155	••••	0.0050	inf	0.10	W_SP	–	–	–	–	–	–	–	–
170	••••	–	–	–	–	0.054	0.14	0.00	W_SP	–	–	–	–
195	••••	–	–	–	–	0.052	0.00	0.062	***S_SP***	–	–	–	–
210	••••	–	–	–	–	–	–	–	–	0.0079	0.16	1.95	***S_SP***
215	••••	0.027	0.00	1.21	***S_SP***	–	–	–	–	0.011	0.00	3.24	***S_SP***
223	••••	–	–	–	–	0.010	0.95	0.00	W_SP	–	–	–	–
231	••••	–	–	–	–	0.019	0.82	0.058	W_SP	–	–	–	–
243	••••	–	–	–	–	0.021	0.00	inf	***S_SP***	–	–	–	–
248	•••• ◆	0.0039	1.58	0.19	W_SP	–	–	–	–	1.16E-05	1.58	0.045	W_SP
250	•••• ◆	–	–	–	–	0.054	0.078	0.87	***S_SP***	–	–	–	–
254	••••	0.036	inf	0.00	W_SP	0.022	0.00	inf	***S_SP***	–	–	–	–
257	••••	–	–	–	–	0.037	0.28	3.07	***S_SP***	–	–	–	–
262	••••	–	–	–	–	0.014	0.00	0.22	***S_SP***	–	–	–	–
263	••••	–	–	–	–	0.023	1.04	0.23	W_SP	0.0092	3.28	0.23	W_SP
265	••••	–	–	–	–	0.011	1.09	0.080	W_SP	0.037	0.88	0.080	W_SP
269	••••	–	–	–	–	–	–	–	–	0.013	inf	0.15	W_SP
270	••••	–	–	–	–	0.052	0.25	1.7E-19	W_SP	–	–	–	–
284	••••	0.038	0.00	0.24	***S_SP***	–	–	–	–	0.026	0.00	0.30	***S_SP***
285	••••	0.0022	inf	0.00	W_SP	–	–	–	–	0.0039	inf	2.6E-17	W_SP
287	••••	0.019	0.00	1.50	***S_SP***	–	–	–	–	–	–	–	–
302	••••	0.013	0.83	0.077	W_SP	–	–	–	–	0.023	0.83	0.081	W_SP
303	••••	–	–	–	–	–	–	–	–	0.0095	inf	0.12	W_SP
311	••••	–	–	–	–	0.032	0.39	3.2E-17	W_SP	–	–	–	–
329	••••	–	–	–	–	0.018	0.48	1.65	***S_SP***	0.017	0.21	1.65	***S_SP***
331	••••	–	–	–	–	0.020	0.52	3.24	***S_SP***	–	–	–	–
335	••••	0.013	0.00	0.72	***S_SP***	–	–	–	–	0.011	0.00	0.76	***S_SP***
349	••••	–	–	–	–	–	–	–	–	0.015	0.31	0.00	W_SP
357	••••	–	–	–	–	–	–	–	–	0.022	0.36	1.5E-15	W_SP
359	••••	–	–	–	–	0.048	0.13	7.79E-16	W_SP	–	–	–	–
366	••••	–	–	–	–	0.029	0.42	0.00	W_SP	–	–	–	–
370	••••	–	–	–	–	0.023	1.97	0.28	W_SP	0.0033	7.24	0.28	W_SP
376	••••	–	–	–	–	0.010	0.19	0.00	W_SP	–	–	–	–
385	••••	–	–	–	–	–	–	–	–	0.025	0.52	0.068	W_SP
393	••••	–	–	–	–	0.018	0.18	inf	***S_SP***	0.0087	0.00	inf	***S_SP***
396	••••	0.0052	inf	0.10	W_SP	–	–	–	–	0.044	inf	0.48	W_SP
418	••••	0.050	inf	0.32	W_SP	–	–	–	–	0.019	inf	0.25	W_SP
426	••••	0.051	0.64	4.81E-16	W_SP	–	–	–	–	0.043	0.64	2.79E-15	W_SP
432	••••	0.0033	inf	0.41	W_SP	–	–	–	–	0.0018	inf	0.19	W_SP
433	••••	0.00074	0.00	2.54	***S_SP***	–	–	–	–	0.051	0.00	0.53	***S_SP***
434	••••	–	–	–	–	–	–	–	–	0.017	inf	0.89	W_SP
445	••••	0.0024	1.41	0.00	W_SP	–	–	–	–	0.039	1.41	0.10	W_SP
451	••••	–	–	–	–	0.047	0.40	0.00	W_SP	–	–	–	–
452	••••	0.040	0.00	inf	***S_SP***	0.026	inf	0.00	W_SP	–	–	–	–
462	••••	0.0035	1.81	0.042	W_SP	–	–	–	–	0.0082	1.81	0.078	W_SP
468	••••	–	–	–	–	0.016	1.14	inf	***S_SP***	–	–	–	–

(Footnotes Table S6.2)

Dif: Difference; dN: rate of non-synonymous substitutions; dS: rate of synonymous substitutions; (H)RI: (Highly) reduced inhibition; inf: infinite (dS=0); NAI: Neuraminidase inhibitor; S_SP: Stronger Selective Pressure; vs: versus; W_SP: Weaker Selective Pressure

Symbol legend:

• N-terminal cytoplasmic domain •• Transmembrane domain ••• Stalk domain •••• Catalytic globular head domain

◆ Epitope contacting residue Associated with (H)RI by NAIs *in vitro* Associated with a synergistic effect on NAI (H)RI

Site-by-site selective pressures (SP) between two different time periods were compared as described in Table 6.5. The dash (-) represents no significant difference, while significant differences involving stronger SP are highlighted in bold and italic.

Table S6.3 Influenza B/Victoria-lineage neuraminidase sites identified as differentially selected between the different time periods analysed (temporal sub-datasets).

Codon site	Site characteristics	Before 1999 (1) *vs* 1999-2008 (2)				1999-2008 (2) *vs* 2009 onwards (3)				Before 1999 (1) *vs* 2009 onwards (3)			
		p value	dN/dS_1	dN/dS_2	DIF	p value	dN/dS_2	dN/dS_3	DIF	p value	dN/dS_1	dN/dS_3	DIF
24	••	–	–	–	–	0.016	inf	0.27	W_SP	–	–	–	–
31	••	0.052	inf	6.69E-16	W_SP	–	–	–	–	0.0022	inf	1.24E-15	W_SP
33	••	–	–	–	–	0.049	0.00	inf	***S_SP***	–	–	–	–
45	•••	–	–	–	–	–	–	–	–	0.035	inf	0.40	W_SP
47	•••	–	–	–	–	0.050	0.00	0.85	***S_SP***	–	–	–	–
51	•••	–	–	–	–	–	–	–	–	0.030	0.072	1.23	***S_SP***
54	•••	–	–	–	–	0.017	0.85	inf	***S_SP***	0.0014	0.13	inf	***S_SP***
75	••••	–	–	–	–	–	–	–	–	0.044	inf	0.48	W_SP
91	••••	0.018	0.00	1.76	***S_SP***	–	–	–	–	–	–	–	–
141	••••	–	–	–	–	0.045	inf	0.034	W_SP	–	–	–	–
152	••••	–	–	–	–	0.054	inf	0.084	W_SP	–	–	–	–
153	••••	–	–	–	–	0.017	1.00	1.77E-15	W_SP	–	–	–	–
187	••••	–	–	–	–	0.047	0.00	inf	***S_SP***	–	–	–	–
199	••••	–	–	–	–	–	–	–	–	0.035	0.33	inf	***S_SP***
202	••••	–	–	–	–	0.034	inf	0.00	W_SP	0.0087	inf	0.00	W_SP
229	••••	–	–	–	–	0.00041	inf	4.49E-18	W_SP	–	–	–	–
237	••••	0.046	0.00	inf	***S_SP***	0.0093	inf	2.43E-17	W_SP	–	–	–	–
246	••••	–	–	–	–	0.049	0.00	inf	***S_SP***	–	–	–	–
247	•••• ◆	–	–	–	–	0.029	0.00	0.56	***S_SP***	–	–	–	–
250	••••	–	–	–	–	0.0030	inf	0.031	W_SP	–	–	–	–
257	••••	–	–	–	–	0.040	0.00	0.74	***S_SP***	–	–	–	–
263	••••	–	–	–	–	–	–	–	–	0.042	0.00	inf	***S_SP***
266	••••	0.032	inf	0.084	W_SP	–	–	–	–	–	–	–	–
282	••••	–	–	–	–	0.034	0.36	0.00	W_SP	–	–	–	–
286	••••	–	–	–	–	0.043	0.58	0.00	W_SP	–	–	–	–
294	•••• □	–	–	–	–	0.053	0.50	0.00	W_SP	–	–	–	–
296	••••	–	–	–	–	0.026	inf	0.00	W_SP	–	–	–	–
304	••••	–	–	–	–	0.054	inf	0.00	W_SP	–	–	–	–
325	••••	–	–	–	–	0.039	0.40	1.96E-15	W_SP	–	–	–	–
338	••••	–	–	–	–	0.013	inf	0.13	W_SP	–	–	–	–
348	••••	–	–	–	–	0.028	0.00	0.72	***S_SP***	–	–	–	–
396	••••	–	–	–	–	–	–	–	–	0.055	inf	0.19	W_SP
416	••••	0.017	0.00	inf	***S_SP***	–	–	–	–	–	–	–	–
434	••••	0.049	inf	0.00	W_SP	–	–	–	–	–	–	–	–
435	•••• ◆	0.051	inf	0.00	W_SP	–	–	–	–	–	–	–	–
436	••••	0.036	inf	0.56	W_SP	–	–	–	–	–	–	–	–
439	••••	0.034	0.00	inf	***S_SP***	0.013	inf	0.19	W_SP	–	–	–	–
449	••••	–	–	–	–	0.024	indet	1.22E-05	–	0.024	indet	1.22E-05	–
459	••••	–	–	–	–	0.033	inf	0.18	W_SP	–	–	–	–
462	••••	–	–	–	–	–	–	–	–	0.017	inf	1.06E-16	W_SP

Dif: Difference; dN: rate of non-synonymous substitutions; dS: rate of synonymous substitutions; (H)RI: (Highly) reduced inhibition; inf: infinite (dS=0); NAI: Neuraminidase inhibitor; S_SP: Stronger Selective Pressure; vs: versus; W_SP: Weaker Selective Pressure

Symbol legend:

•• Transmembrane domain ••• Stalk domain •••• Catalytic globular head domain

□ Framework residue NA active site ◆ Potential epitope contacting residue (antibody escape mutants) Associated with (H)RI by NAIs *in vitro*

Site-by-site selective pressures (SP) between two different time periods were compared as described in Table 6.5. The dash (-) represents no significant difference, while significant differences involving stronger SP are highlighted in bold and italic.

Table S6.4 Influenza B/Yamagata-lineage neuraminidase sites identified as differentially selected between the different time periods analysed (temporal sub-datasets).

Codon site	Site characteristics	Before 1999 (1) *vs* 1999-2008 (2)				1999-2008 (2) *vs* 2009 onwards (3)				Before 1999 (1) *vs* 2009 onwards (3)			
		p value	dN/dS_1	dN/dS_2	DIF	p value	dN/dS_2	dN/dS_3	DIF	p value	dN/dS_1	dN/dS_3	DIF
12	•	–	–	–	–	0.035	0.00	inf	***S_SP***	0.047	0.00	inf	***S_SP***
26	••	0.050	0.19	inf	***S_SP***	–	–	–	–	–	–	–	–
39	••	–	–	–	–	0.034	inf	0.00	W_SP	–	–	–	–
41	••	–	–	–	–	–	–	–	–	0.033	0.00	inf	***S_SP***
54	•••	0.019	0.00	inf	***S_SP***	–	–	–	–	–	–	–	–
57	•••	–	–	–	–	0.025	0.00	inf	***S_SP***	–	–	–	–
58	•••	–	–	–	–	–	–	–	–	0.050	inf	0.00	W_SP
72	••••	–	–	–	–	0.017	0.15	inf	***S_SP***	–	–	–	–
76	••••	–	–	–	–	0.052	0.00	0.93	***S_SP***	–	–	–	–
78	••••	0.050	0.00	inf	***S_SP***	–	–	–	–	–	–	–	–
104	••••	–	–	–	–	0.051	inf	0.00	W_SP	–	–	–	–
106	••••	–	–	–	–	–	–	–	–	0.036	0.57	inf	***S_SP***
109	••••	–	–	–	–	0.050	0.00	inf	***S_SP***	0.049	0.00	inf	***S_SP***
125	••••	–	–	–	–	0.012	2.13	0.22	W_SP	–	–	–	–
148	••••	–	–	–	–	0.051	inf	0.00	W_SP	–	–	–	–
154	•••• □	–	–	–	–	0.050	0.00	inf	***S_SP***	–	–	–	–
172	••••	–	–	–	–	0.0093	0.00	inf	***S_SP***	0.049	0.00	inf	***S_SP***
194	••••	–	–	–	–	0.052	inf	0.00	W_SP	–	–	–	–
197	•••• □ ↓	–	–	–	–	0.050	inf	0.32	W_SP	–	–	–	–
224	••••	–	–	–	–	0.050	inf	0.00	W_SP	0.051	inf	0.00	W_SP
248	••••	–	–	–	–	0.018	inf	0.45	W_SP	–	–	–	–
290	••••	–	–	–	–	0.020	0.00	0.69	***S_SP***	–	–	–	–
342	•••• ◆	–	–	–	–	–	–	–	–	0.022	0.00	0.64	***S_SP***
345	••••	–	–	–	–	–	–	–	–	0.021	inf	0.16	W_SP
371	••••	–	–	–	–	0.033	0.00	inf	***S_SP***	–	–	–	–
384	••••	–	–	–	–	0.014	0.00	inf	***S_SP***	–	–	–	–
404	••••	0.043	0.074	inf	***S_SP***	–	–	–	–	–	–	–	–
436	••••	0.022	inf	0.75	W_SP	–	–	–	–	0.014	inf	0.73	W_SP
443	••••	0.050	0.00	inf	***S_SP***	–	–	–	–	–	–	–	–
463	••••	0.031	0.00	1.38	***S_SP***	–	–	–	–	0.031	0.00	1.06	***S_SP***

Dif: Difference; dN: rate of non-synonymous substitutions; dS: rate of synonymous substitutions; (H)RI: (Highly) reduced inhibition; inf: infinite (dS=0); NAI: Neuraminidase inhibitor; S_SP: Stronger Selective Pressure; vs: versus; W_SP: Weaker Selective Pressure

Symbol legend:

• N-terminal cytoplasmic domain •• Transmembrane domain ••• Stalk domain •••• Catalytic globular head domain

□ Framework residue NA active site ◆ Potential epitope contacting residue (antibody escape mutants) ↓ Associated with (H)RI by NAIs *in vitro*

Site-by-site selective pressures (SP) between two different time periods were compared as described in Table 6.5. The dash (-) represents no significant difference, while significant differences involving stronger SP are highlighted in bold and italic.

Table S6.5 2009 pandemic N1 neuraminidase sites identified as differentially selected between the different time periods analysed (temporal sub-datasets).

Codon site	Site characteristics	p value	dN/dS		DIF
			(Pre-)Pandemic period	Post-pandemic period	
4	•	0.012	0.45	0.076	W_SP
13	••	0.041	0.70	2.67	***S_SP***
44	•••	0.022	0.33	1.29	***S_SP***
50	•••	0.031	5.21E-07	0.51	***S_SP***
67	•••	0.0075	0.071	0.68	***S_SP***
69	•••	0.0074	0.084	2.83	***S_SP***
93	•••• ◆	0.0031	0.146	2.18	***S_SP***
117	••••	0.0024	0.00	inf	***S_SP***
136	•••• ↓	0.00090	0.00	2.30	***S_SP***
145	••••	0.022	0.00	0.73	***S_SP***
167	••••	0.047	0.00	0.24	***S_SP***
170	••••	0.0062	0.00	0.46	***S_SP***
181	••••	0.042	0.30	0.00	W_SP
220	•••• ◆	0.022	0.20	1.33	***S_SP***
247	•••• ↓ ↓	0.047	2.26	inf	***S_SP***
295	•••• □ ↓	0.019	0.00	0.22	***S_SP***
321	••••	0.0081	0.32	4.20	***S_SP***
324	••••	0.047	7.40E-17	1.13	***S_SP***
369	••••	0.051	1.53	0.35	W_SP
388	•••• ◆	0.038	1.24	0.15	W_SP
406	••••	0.053	inf	0.00	W_SP
440	••••	0.041	1.81E-15	0.23	***S_SP***
443	••••	0.0075	0.067	inf	***S_SP***
447	••••	0.028	0.00	0.81	***S_SP***
461	••••	0.034	0.00	inf	***S_SP***
462	••••	0.024	1.92	0.00	W_SP

Dif: Difference; dN: rate of non-synonymous substitutions; dS: rate of synonymous substitutions; (H)RI: (Highly) reduced inhibition; inf: infinite (dS=0); NAI: Neuraminidase inhibitor; S_SP: Stronger Selective Pressure; W_SP: Weaker Selective Pressure

Symbol legend:

• N-terminal cytoplasmic domain •• Transmembrane domain ••• Stalk domain •••• Catalytic globular head domain

□ Framework residue NA active site ◆ Epitope contacting residue ↓ Associated with (H)RI by NAIs *in vitro* ↓ Associated with a synergistic effect on NAI (H)RI

Site-by-site selective pressures (SP) were compared as described in Table 6.5. Significant differences involving stronger SP are highlighted in bold and italic.

CONCLUDING REMARKS

This research project has made possible to have, at national level, a solid and up-to-date platform for phenotypic and/or genotypic evaluation of influenza virus susceptibility to antiviral drugs. National antiviral susceptibility data was gathered over a 10-year period (2004-2013), providing comprehensive information and experience on the susceptibility of circulating human influenza viruses and on its natural variation across the different virus types or subtypes and over time. It is essential to continue monitoring influenza virus susceptibility in following winter seasons, even more when recent worldwide data suggests a potential risk for the occurrence of seasonal epidemics of neuraminidase (NA) H275Y A(H1N1)pdm09 drug-resistant viruses and the selective pressure data here obtained further supports that (evidence of positive directional selection). In these following testing activities, it will be important to include the two novel NA inhibitors (NAIs) peramivir and laninamivir that, although only available in limited markets, have potential for worldwide approval. Both drugs were already received in the laboratory for testing. Enhanced efforts to ensure that more specimens from patients undergoing antiviral therapy are collected will also be important. This will not only allow to determine the frequency of resistance or reduced susceptibility emerging as a result of treatment, but also to identify which shifts in IC_{50} are clinically relevant and help determining the IC_{50} cut off for clinical relevance in NA inhibition assays. The technological capacity and know-how now available also allow us to advance to a more specific line of research focused on elucidating the role of NA amino acid substitutions in NAI susceptibility, as those here identified: R222Q and D344N in seasonal N1 NA; D151N, S367N and K369T in N2 NA; I240V, K219N and K373E in B/Victoria-lineage NA. It will also be interesting to expand the study to hemagglutinin (HA) and elucidate the role of H3 HA T135A, R142G and S262N, and 2009 H1 HA D222G amino acid substitutions. However, for this it will be necessary to establish an *in vitro* susceptibility assay accounting for HA-mediated changes, most probably a red blood cell (RBC) elution assay.

Whole-genome sequencing did not reveal any possible explanation for why NA H275Y drug-resistant former seasonal A(H1N1) viruses were fitter and spread over their sensitive counterparts. However, further *in vitro* and *in vivo* studies are needed to confirm that the PB2 P453S and PB1 N642S amino acid substitutions found specifically in their genome have no effect on virus fitness, as suggested by their location onto the

protein structure. The identification of amino acid substitutions known to or that based on their functional impact may enhance viral fitness in influenza viruses resistant or with decreased susceptibility to NAIs, highlighted the importance of extending genetic analysis to all segments of influenza genome.

Selective pressure (SP) analysis was mainly focused on the NA sites associated with resistance or reduced susceptibility to NAIs and/or contacting with the drug, but it also allowed a comprehensive study of the global and site-specific SP acting on human influenza NA. Knowing now that most sites associated with NAI resistance or reduced susceptibility are either negatively selected or experiencing a n.s. $dN/dS<1$, it will be interesting to investigate the number and type of amino acid changes that have to occur for circulating viruses become resistant or possess (H)RI *in vitro* – genetic barrier to resistance or (H)RI. Also, there is a strong possibility of further studying the 6 unknown regions here identified as potential new druggable targets in the context of a new PhD research project now initiated in the laboratory, focused on exploring NS1 as a potential emerging antiviral target.

It was a true pleasure

THE END

APPENDICES

APPENDIX A

Journal of Medical Virology 87:45–56 (2015)

Influenza A(H1N1)pdm09 Resistance and Cross-Decreased Susceptibility to Oseltamivir and Zanamivir Antiviral Drugs

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Neuraminidase inhibitors (NAIs) oseltamivir and zanamivir are currently the only effective antiviral drugs available worldwide for the management of influenza. The potential development of resistance is continually threatening their use, rationalizing and highlighting the need for a close and sustained evaluation of virus susceptibility. This study aimed to analyze and characterize the phenotypic and genotypic NAIs susceptibility profiles of A(H1N1)pdm09 viruses circulating in Portugal from 2009 to 2010/2011. A total of 144 cases of A(H1N1)pdm09 virus infection from community and hospitalized patients were studied, including three suspected cases of clinical resistance to oseltamivir. Oseltamivir resistance was confirmed for two of the suspected cases. Neuraminidase (NA) H275Y resistant marker was found in viruses from both cases but for one it was only present in 26.2% of virus population, raising questions about the minimal percentage of resistant virus that should be considered relevant. Cross-decreased susceptibility to oseltamivir and zanamivir (2–4 IC_{50} fold-change) was detected on viruses from two potentially linked community patients from 2009. Both viruses harbored the NA I223V mutation. NA Y155H mutation was found in 18 statistical non-outlier viruses from 2009, having no impact on virus susceptibility. The mutations at NA N369K and V241I may have contributed to the significantly higher baseline IC_{50} value obtained to oseltamivir for 2010/2011 viruses, compared to viruses from the pandemic period. These results may contribute to a better understanding of the relationship between phenotype and genotype, which is currently challenging, and to the global assessment of A(H1N1)pdm09 virus susceptibility profile and baseline level to NAIs. ***J. Med. Virol. 87:45–56, 2015.***

KEY WORDS: A(H1N1) 2009 pandemic variant; neuraminidase inhibitors; susceptibility testing; NA H275Y; NA I223V; Portugal

INTRODUCTION

A(H1N1)2009 pandemic was the first influenza pandemic having the assistance of an extensive preparedness and response planning. Advance stockpiling of antiviral drugs was one of the key measures recommend by World Health Organization (WHO), and neuraminidase inhibitors (NAIs), in particular oseltamivir, were the antiviral drugs of choice for many developed countries [WHO, 2005, 2009a; Meijer et al., 2007; Oshitani et al., 2008]. Portugal stockpiled a large amount of oseltamivir phosphate (enough for 2.51 million treatments and 100,000 prophylactic schemes) and a small quantity of zanamivir (enough for 42 treatments). This national stockpile was released on April 24, 2009, immediately after the activation of the National Contingency Plan due to A(H1N1)pdm09 virus emergence [DGS, 2010]. National progression from the containment to mitigation pandemic stage on 21st August and availability of the pandemic vaccine on 26th October required timely national updates on the recommendations for antiviral drug use during 2009 [DGS, 2010].

NAIs were an appropriate choice for A(H1N1)2009 pandemic prevention and control. This new variant of the *Orthomyxoviridae* family, *Influenzavirus A* genus, exhibited natural resistance to M2 protein inhibitors and clinical effectiveness of NAIs was reported in observational studies carried out during the pandemic period [CDC, 2009; Dominguez-Cherit et al., 2009; Jain et al., 2009; Pada and Tambyah, 2011]. Also, the emergence of resistant variants was only detected for oseltamivir and limited to sporadic events, largely in association with drug use and patients with an immunocompromised condition [WHO, 2011a]. The first case of oseltamivir resistance was reported by Denmark Health Authorities 3 months after the emergence of the A(H1N1)2009 variant, demonstrating the importance of national capability for the rapid detection of resistant variants [ECDC, 2009]. Since then and until October 5, 2011, a further 604 cases of oseltamivir resistance were detected globally, all harboring the neuraminidase (NA) H275Y mutation. Person-to-person transmission of oseltamivir-resistant virus was identified during the pandemic period but only in three limited clusters, variable in size, timing and location [WHO, 2010, 2011a]. During the following 2010/2011 season, national surveillance activities carried out at the United Kingdom, Japan, and United States reported an increase in the proportion of A(H1N1)pdm09 oseltamivir-resistant virus with no known exposure to the drug, potentially indicating a low-level transmission of resistant virus in the community [Lackenby et al., 2011; WHO, 2011a]. This was confirmed recently, by the identification of a community cluster of 6 A(H1N1)pdm09 oseltamivir and peramivir-resistant viruses (H275Y) with no known association to drug use during the end of 2013 in Japan [Takashita et al., 2014]. Regarding zanamivir, only one case of reduced susceptibility was identified to date, in a virus harboring NA I223R mutation collected from an immunocompromised child [WHO, 2011a].

The aim of this study was to analyze and characterize the NAIs susceptibility profiles of influenza A(H1N1)pdm09 viruses circulating in Portugal, from the 2009 pandemic period to the 2010/2011 winter season and in both community and hospital settings, to NAIs (oseltamivir and zanamivir). Suspected cases of clinical resistance development were also studied.

MATERIALS AND METHODS

Clinical Specimens and Virus Strains

Clinical specimens from the upper respiratory tract of patients positive for influenza A(H1N1)pdm09 virus infection were used. Specimens were provided by two national reference hospitals—Hospital de Curry Cabral (HCC) and Hospital Dona Estefânia (HDE) (Lisbon, Portugal). Specimen collection was performed in hospitalized and in community patients attending the hospital emergency unit, from the 2009 pandemic period (June 2009–January 2010) to the 2010/2011 winter season (January–March 2011).

A total of 216 cases of influenza A(H1N1)pdm09 virus infection, 163 from the pandemic period and 53 from 2010/2011, were selected for study. This includes three suspected cases of clinical resistance identified by the hospital clinicians during 2010/2011 in non-related hospitalized patients that failed to respond to oseltamivir treatment (positive for influenza after >10 days of therapy), independently of the timing between symptom onset and initiation of therapy (< or >48 hr) (Table I). Clinical specimens from these three suspected cases were directly tested for the presence of the NA H275Y oseltamivir-resistant marker. These and all the other 213 specimens from the remaining selected cases underwent viral isolation in MDCK-SIAT1 cells. A total of 142 A(H1N1)pdm09 virus strains were isolated successfully, 116 from the pandemic period and 26 from 2010/2011, and evaluated further for phenotypic antiviral drug susceptibility, using mainly a second or third cell passage isolate. This includes the virus strain isolated from suspected case 2 (A/Portugal/03/2011), that was the only suspected case for which viral isolation was successful (Table I). Fifty of the 142 virus strains tested phenotypically, 44 from the pandemic period and 6 from 2010/2011, were evaluated for genotypic antiviral drug susceptibility by NA and hemagglutinin (HA) sequencing. This comprised all virus strains identified as statistical outliers in the phenotypic evaluation and approximately 25% of the non-outlier strains (randomly selected).

Overall, 144 of the total selected 216 cases of influenza A(H1N1)pdm09 virus infection were studied. Two cases were only studied genotypically for the specific presence of H275Y mutation (suspected cases 1 and 3), while the remaining 142 cases (including suspected case 2) were completely studied through phenotypic and/or genotypic analysis.

Screening Assay for Rapid Detection of NA H275Y Oseltamivir-Resistant Marker

Viral extraction was performed using the automated extractor EasyMag (bioMérieux, Linda-a-Velha, Portugal) according to the manufacturer's recommendations. The presence of NA H275Y oseltamivir-resistant marker was searched by real-time RT-PCR, using a protocol kindly provided by Prof. Martin

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TABLE I. Epidemiological, Clinical, and Laboratorial Information Concerning the Three Cases From 2010/2011 Suspected of Clinical Resistance Development to Oseltamivir

Case number	1	2	3
Epidemiological and clinical information			
Patient			
Age (years)	1	27	61
Gender	Male	Female	Male
Underlying risk conditions	Premature bronchopulmonary dysplasia (hospitalized since birth)	HIV+; pregnant	Not referred
Clinical disease	Severe	Severe with loss of the fetus	Severe
Influenza antiviral drug treatment			
Antiviral drug	Oseltamivir	Oseltamivir	Oseltamivir
Starting date	11 days after the 1st positive rt RT-PCR	Not referred	Not referred
Evidence of failure	rt RT-PCR positive after 15 days of treatment	Not referred	rt RT-PCR positive after 10 days of treatment
Time between the start of antiviral therapy and specimen collection	20 days	Not possible to estimate	≥10 days
Clinical outcome	Not referred	Deceased	Not referred
Other observations	—	Initiated treatment with zanamivir; from Guinea (Africa)	—
Clinical specimen laboratorial results			
H275Y oseltamivir-resistant marker	Not present	Present (Ct = 13)	Present as quasi-species (Ct = 27 for 275Y; Ct = 28 for 275H), representing 26.2% of the virus population
Virus strain isolated	No strain isolated	A/Portugal/03/2011	No strain isolated

Curran (Public Health England [PHE], Addenbrooke's Hospital, Cambridge, UK). These methodologies were performed at HCC.

Quantification of H275Y Oseltamivir-Resistant Quasi-Species

The proportion of wild-type 275H and mutant 275Y virus in oseltamivir-resistant quasi-species was quantified by pyrosequencing. This method was performed at the PHE (Colindale, London, UK), following the standard operating procedure in use [HPA, 2010].

Genotypic Evaluation of Virus Susceptibility to NAIs

Genotypic evaluation was performed by NA and HA full-length gene sequencing. RNA extraction was performed with QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany). Sequencing methodology and analysis included the CDC protocol recommended by WHO [2009b] and the use of SeqMan application of Lasergene software v.4.05 (DNASTAR, Inc., Madison, WI), as described previously [Santos et al., 2011; Giria et al., 2012]. MEGA5 software was used for multiple sequence alignment by Clustal W method and for mutational analysis against A/California/07/2009 vaccine strain sequences. N1 numbering was used for NA and the signal peptide sequence was not considered for HA numbering. Phylogenetic analysis included (1) determination of the best-fit model for nucleotide substitution according to Akaike Information Criterion, using jModelTest v.2.1.2 [Guindon and Gascuel, 2003; Darriba et al., 2012]; and (2) construction of phylogenetic trees by maximum-likelihood method using PhyML 3.0 software available on Seaview multiplatform v.4.4.0. Approximate likelihood-ratio tests (aLRT(SH-like)) were used for measuring branch support and subtree-pruning-and-regrafting (SPR) and nearest-neighbor-interchange (NNI) rearrangement operations were considered for tree topology improvement. NA and HA sequences of A/California/07/2009 vaccine strain and of reference strains were included for comparative analysis. These sequences were retrieved from the Global Initiative on Sharing All Influenza Data (GISAID) EpiFlu™ Database and from the National Center for Biotechnology Information Influenza Virus Resource. Accession numbers are provided in the phylogenetic trees, after the sequence name. In addition, the accession numbers from the sequences downloaded from GISAID database are listed in Table II, to acknowledge the sequence providers.

Phenotypic Evaluation of Virus Susceptibility to NAIs

Phenotypic profiling was based on IC_{50} determination, using the new WHO IC_{50} fold-change criteria that for influenza A viruses is: fold-change increase <10—normal inhibition (NI); 10–100 fold-change increase—reduced inhibition (RI); fold-change increase >100—highly reduced inhibition (HRI) [Hurt et al., 2012; WHO, 2012]. Non-outlier IC_{50} values from A(H1N1) seasonal strains that circulated in Portugal

TABLE II. Origin of the A(H1N1)pdm09 Reference Sequences Downloaded From GISAID EpiFlu™ Database Included in the Neuraminidase (NA) and Hemagglutinin (HA) Sequence Alignments for Comparative Analysis

Segment ID	Segment	Country	Collection date	Isolate name	Originating lab	Submitting lab
EPI239630	NA	Germany	January 1, 2009	A/Bayern/69/2009		Centers for Disease Control and Prevention
EPI239631	HA	Germany	January 1, 2009	A/Bayern/69/2009		Centers for Disease Control and Prevention
EPI215956	NA	Ukraine	October 27, 2009	A/Lviv/N6/2009	Ministry of Health of Ukraine	National Institute for Medical Research
EPI215957	HA	Ukraine	October 27, 2009	A/Lviv/N6/2009	Ministry of Health of Ukraine	National Institute for Medical Research
EPI280343	NA	New Zealand	July 12, 2010	A/Christchurch/16/2010	WHO Collaborating Centre for Reference and Research on Influenza	Centers for Disease Control and Prevention
EPI280344	HA	New Zealand	July 12, 2010	A/Christchurch/16/2010	WHO Collaborating Centre for Reference and Research on Influenza	Centers for Disease Control and Prevention
EPI279896	NA	Hong Kong (SAR)	July 16, 2010	A/Hong Kong/2213/2010	Government Virus Unit	National Institute for Medical Research
EPI279897	HA	Hong Kong (SAR)	July 16, 2010	A/Hong Kong/2213/2010	Government Virus Unit	National Institute for Medical Research
EPI319498	NA	France	December 31, 2010	A/Lyon/3/2010	CRR virus Influenza region Sud	National Institute for Medical Research
EPI319497	HA	France	December 31, 2010	A/Lyon/3/2010	CRR virus Influenza region Sud	National Institute for Medical Research
EPI319591	NA	Russian Federation	February 28, 2011	A/Astrakhan/1/2011	WHO National Influenza Centre	National Institute for Medical Research
EPI319590	HA	Russian Federation	February 28, 2011	A/Astrakhan/1/2011	WHO National Influenza Centre	National Institute for Medical Research
EPI319448	NA	Czech Republic	January 18, 2011	A/Czech Republic/32/2011	National Institute of Public Health	National Institute for Medical Research
EPI319447	HA	Czech Republic	January 18, 2011	A/Czech Republic/32/2011	National Institute of Public Health	National Institute for Medical Research
EPI331060	NA	Ghana	April 14, 2011	A/Ghana/601/2011	University of Ghana	National Institute for Medical Research
EPI331059	HA	Ghana	April 14, 2011	A/Ghana/601/2011	University of Ghana	National Institute for Medical Research
EPI326207	NA	Hong Kong (SAR)	March 29, 2011	A/Hong Kong/3934/2011	Government Virus Unit	National Institute for Medical Research
EPI326206	HA	Hong Kong (SAR)	March 29, 2011	A/Hong Kong/3934/2011	Government Virus Unit	National Institute for Medical Research
EPI319528	NA	Russian Federation	February 14, 2011	A/St. Petersburg/27/2011	WHO National Influenza Centre	National Institute for Medical Research
EPI319527	HA	Russian Federation	February 14, 2011	A/St. Petersburg/27/2011	WHO National Influenza Centre	National Institute for Medical Research
EPI320140	NA	Russian Federation	March 14, 2011	A/St. Petersburg/100/2011	Russian Academy of Medical Sciences	Centers for Disease Control and Prevention
EPI320141	HA	Russian Federation	March 14, 2011	A/St. Petersburg/100/2011	Russian Academy of Medical Sciences	Centers for Disease Control and Prevention

We acknowledge the authors, originating and submitting laboratories of the sequences from GISAID's EpiFlu™ Database on which this research is based. All submitters of data may be contacted directly via the GISAID website www.gisaid.org.

from 2004/2005 to 2008/2009 (N = 59 for oseltamivir; N = 70 for zanamivir) [Correia et al., 2010] were used for performing IC_{50} A(H1N1) lineage comparative analysis.

Phenotypic Assay

Phenotypic testing was performed using the in-house MUNANA-based IC_{50} fluorescence assay provided by PHE [HPA, 2006], as described previously in Correia et al. [2010]. Oseltamivir carboxylate and zanamivir were provided through a material transfer agreement by, respectively, F.Hoffmann-La Roche Ltd (Basel, Switzerland) and GlaxoSmithKline (Hertfordshire, UK).

Statistical Analysis

IC_{50} values were determined through point-to-point analysis using Microsoft Office Excel 2007. Statistical outliers were identified by determination of upper and lower cut offs, with each one including a mild (1.65 standard deviations [SD] above or below the median, respectively) and an extreme (3 SD above or below the median, respectively) level. Cut offs were determined using Scaled Median Absolute Deviation (SMAD) statistic method [AMC, 2001]. All outlier strains were retested twice and the mean IC_{50} value was considered for analysis. Median baseline (median without upper and lower outliers) was used for determination of the fold-change thresholds – RI (median × 10) and HRI (median × 100), and of the individual fold-changes. Linear regression for time trend analysis was performed using the Microsoft Office Excel 2007 Analysis ToolPak. Cut offs, median baselines, fold-change thresholds and time trends were determined separately for each time period analyzed. Independent sample's t and paired sample's t-tests were performed in SPS Statistics software v17.0 and a P-value <0.05 was considered as statistically significant. All phenotypic data were log-transformed before being used for statistical analysis.

RESULTS

H275Y Mutation Analysis in Clinical Suspected Resistant Cases

The presence of NA H275Y mutation was detected in the clinical specimens of both cases 2 and 3, confirming the suspicion of clinical resistance development to oseltamivir. In the specimen of suspected case 1, only wild-type 275H virus was detected by amplification. Detailed results are presented in Table I.

Virus Susceptibility Phenotype to NAIs

Oseltamivir. A total of 126 (88.7%; N = 142) A(H1N1)pdm09 virus strains, 104 (89.7%; N = 116) from the pandemic period and 23 (88.5%; N = 26) from 2010/2011, exhibited an IC_{50} value within the normal range to oseltamivir (range of values between mild lower and upper cut offs—median ±1.65 SD). The IC_{50} values varied from 0.50 to 1.07 nM for pandemic period strains and from 0.69 to 1.21 nM for 2010/2011 strains. The other 15 (10.6%) strains, 12 (10.3%) from the pandemic period and 3 (11.5%) from 2010/2011, were identified as statistical outliers. Nine (6.3%) with IC_{50} values falling over upper cut offs, 4 at extreme level and 5 at mild level, and 6 (5.2%) with IC_{50} values falling under lower cut offs (1 extreme and 5 mild). Figure 1A gives information on oseltamivir outlier strains and IC_{50} fold-changes including all IC_{50} values obtained for oseltamivir, statistically analyzed and distributed by time. A/Portugal/03/2011 (isolated from H275Y-confirmed case 2) was the only strain exhibiting a HRI phenotypic profile to oseltamivir, with an ~250 fold-change increase in the IC_{50} value (2010/2011 RI threshold = 8.85; 2010/2011HRI threshold = 88.50). Despite detection of statistical outliers, all the other 141 strains analyzed showed a NI phenotype to this antiviral drug.

No apparent time grouping was observed for upper and/or lower statistical outliers, with exception of the two upper extreme outliers from 2009 (A/Portugal/17/2009 and A/Portugal/82/2009), which are from 2 successive weeks (Fig. 1A). These two outlier strains are from two A(H1N1)pdm09 positive cases closely related at the geographic level but without epidemiological link.

Considering non-outlier IC_{50} values, a significant decreasing trend over time was observed among the 2009 pandemic period values ($P = 0.00516$, $R^2 = 0.074172$, linear regression fit). However, the very low R^2 value obtained indicates that time is a poor predictor of IC_{50} variation. A significant difference was also observed when comparing the non-outlier IC_{50} values from the two time periods analyzed ($P = 0.001$, independent sample's t-test).

Zanamivir. For zanamivir, 131 (92.3%, N = 142) virus strains, 110 (94.8%; N = 116) from the pandemic period and 21 (80.8%; N = 26) from 2010/2011, exhibited an IC_{50} value within the normal range. Specifically, the IC_{50} values from pandemic period strains varied from 0.40 to 0.99 nM and those from 2010/2011 strains varied from 0.41 to 0.80 nM. The other 11 (7.7%) strains, 6 (5.2%) from the pandemic period and 5 (19.2%) from 2010/2011, were identified as statistical outliers. Six (5.2%) with IC_{50} values falling over upper cut offs, one at extreme level and five at mild level, and five (3.5%) with IC_{50} values falling under lower cut offs (one extreme and four mild). Figure 1B shows information on zanamivir outlier strains and IC_{50} fold-changes including all zanamivir IC_{50} values, statistically analyzed and distributed by time. The strains A/Portugal/17/2009, A/Portugal/82/2009, and A/Portugal/07/2011 exhibited to zanamivir the same phenotype displayed to oseltamivir, of upper outlier. Despite detection of statistical outliers, all the 142 A(H1N1)pdm09 analyzed strains shared a NI phenotypic profile to zanamivir (RI fold-change

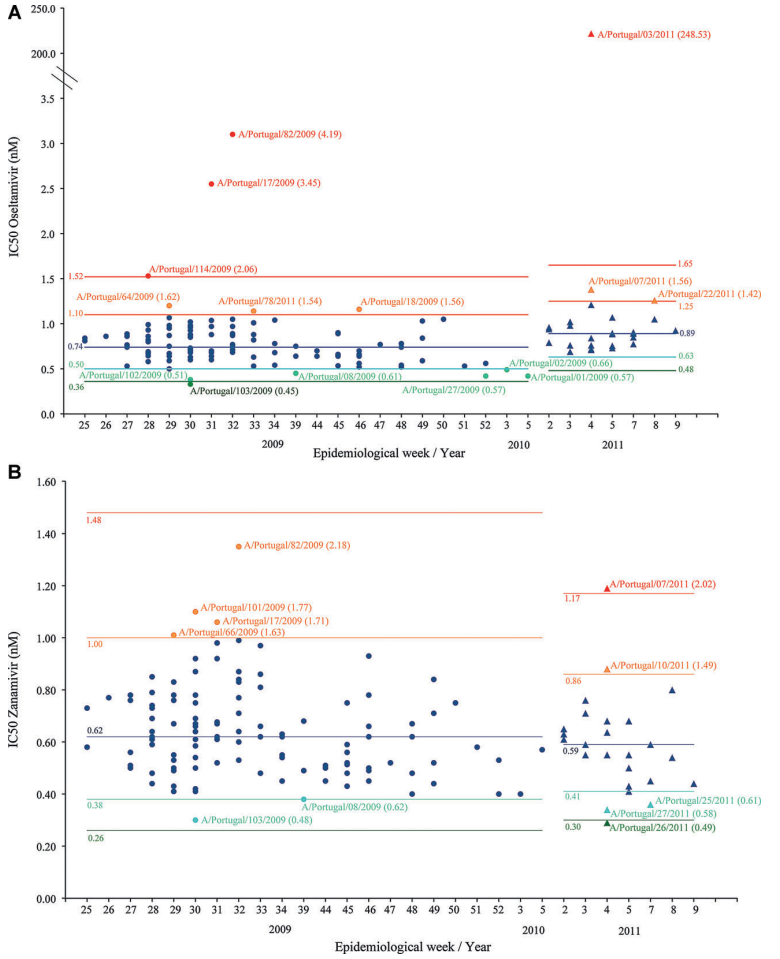

Fig. 1. SMAD statistical analysis of the IC_{50} values obtained for the 142 A(H1N1)pdm09 virus strains analyzed phenotypically, 116 from the pandemic period and 26 from the 2010/2011 season, displayed by week of specimen collection, for oseltamivir (**A**) and zanamivir (**B**). The IC_{50} values of virus strains from the pandemic period are coloured in black and those of virus strains from 2010/2011 are coloured in white (colored web version: circles represent the IC_{50} values of virus strains from the pandemic period and triangles the IC_{50} values of virus strains from 2010/2011). The IC_{50} fold-change observed in relation to median baseline is indicated in brackets after the strain designation.

threshold = 6.20 [pandemic period] or 5.90 [2010/2011]; HRI fold-change threshold = 62.0 [pandemic period] or 59.0 [2010/2011]).

Time analysis revealed that upper outliers from the pandemic period grouped between weeks 29 and 32 of 2009, but no epidemiological link was found (Fig. 1B).

Concerning non-outlier IC_{50} values, a significant decreasing trend over time was detected for 2009 pandemic period values ($P = 0.00481$, $R^2 = 0.071277$, linear regression fit). However, as observed for oseltamivir, the very low R^2 value prevents interpretation of the causal relationship between these two variables. No significant difference was observed between the values from the two time periods analyzed ($P = 0.374$, independent sample's t-test).

Oseltamivir and zanamivir non-outlier IC_{50} values differed significantly ($P = 0.000$, paired sample's t-test). A significant difference was also observed for both NAIs when comparing the non-outlier IC_{50} values from the A(H1N1)pdm09 strains analyzed here with those from A(H1N1) seasonal strains that circulated at national level from 2004/2005 to 2008/2009 ($P = 0.000$, independent sample's t-test) (Fig. 2).

A total of 12 A(H1N1)pdm09 virus strains (8 from the pandemic period and 4 from 2010/2011) were classified as upper statistical outliers for oseltamivir and/or zanamivir and thus selected for further investigation at genotypic level. From these, only A/Portugal/03/2011 had a known association to drug use, having been isolated from suspected case 2 clinical specimen.

Virus Susceptibility Genotype to NAIs

NA and HA amino acid mutations found specifically in the sequences of the 12 phenotypic upper outlier strains are indicated and characterized in Table III.

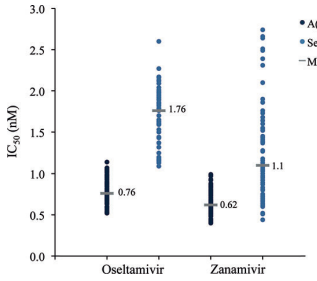

Fig. 2. Comparative analysis of oseltamivir and zanamivir non-outlier IC_{50} values obtained for the A(H1N1)pdm09 virus strains analyzed in this study (N = 126 oseltamivir; N = 132 zanamivir) and for seasonal A(H1N1) virus strains previously circulating at national level, from 2004/2005 to 2008/2009 (N = 59 oseltamivir; N = 70 zanamivir).

NA-specific mutations of oseltamivir extreme outlier strains A/Portugal/17/2019, A/Portugal/82/2009 and A/Portugal/03/2011, have a known reduced susceptibility profile (I223V and H275Y). Only A/Portugal/03/2011 exhibited specific mutations in the HA sequence, harboring an N156D mutation, located in an antigenic site. Regarding all the other oseltamivir and/or zanamivir outlier strains, no shared mutation or known association to a reduced susceptibility profile were identified. In addition, approximately half of these outliers lacked any specific mutations at NA or HA sequences (Table III).

Mutation NA Y155H, associated with a HRI phenotypic profile to both NAIs in seasonal A(H1N1) subtype [Nguyen et al., 2012], was found in the sequence of 18 non-outlier A(H1N1)pdm09 strains from 2009 (12.7%; 18/142), having no apparent impact on NAIs phenotypic susceptibility (Fig. 3A).

Phylogenetic analysis of NA and HA nucleotide sequences revealed two important findings: (1) lack of nucleotide differences between the sequences of the two upper outlier strains A/Portugal/17/2009 and A/Portugal/82/2009, with pairwise distance calculations confirming the 100% identity (results not shown); and (2) no clustering among all the other outlier strains, with outlier sequences being closely related to non-outlier sequences from the same time period (Fig. 3A and B).

DISCUSSION

This study comprised the analysis of A(H1N1) pdm09 virus collected from community and from hospitalized patients, with and without antiviral drug treatment. Using this integrated approach it was possible to cover the two possible settings where influenza antiviral drug resistance or reduced susceptibility can emerge—spontaneously in the community or associated with drug use.

Oseltamivir resistance was identified for two A(H1N1)pdm09 cases from 2010/2011, both from oseltamivir-treated patients (suspected cases 2 and 3). The NA H275Y resistant marker was found in viruses from both cases but for one (suspected case 3) it was only detected in 26.2% of the clinical specimen viral population. The fact that this percentage of resistant virus could have been sufficient for causing treatment failure raised questions about the minimal percentage of resistant virus that should be considered relevant in quasi-species. For suspected case 2, the NA H275Y mutation was identified in the entire viral population of both clinical specimen and virus isolate (A/Portugal/03/2011 strain). Phenotypic analysis of this virus strain revealed a HRI profile to oseltamivir ($IC_{50} = 219.95$ mM; ~250 fold-change reduction). When compared with H275Y oseltamivir-resistant A(H1N1) seasonal strains that circulated previously at national level ($IC_{50} = 314.04–1208.84$ mM; 159–613 fold-change) [Correia et al., 2010], this A(H1N1)pdm09 strain exhibited a lower IC_{50} value and

TABLE III. Listing and Characterization of the Mutations Found Specifically in the Neuraminidase (NA) and Hemagglutinin (HA) Protein Sequences of the Phenotypic Upper Outlier Virus Strains

Protein	Virus strain: Designation	Virus strain: Phenotype (IC_{50} fold-change)	Amino acid change	Polarity change	Effect on N-linked glycosylation	Location in protein structure[d]	Association with reduced susceptibility to NAIs[e]
NA (N1 numbering)	A/Portugal/17/2009	UEOO/NI (3.45) + UMOZ/NI (1.71)	I223V	No	No	Global head, Framework residue of active site	Yes—increase in oseltamivir IC_{50} values for N1 viruses (NI) and for A(H3N2) and B viruses (HRI). Strongly with H275Y in N1 viruses for oseltamivir and peramivir and with E119V in A(H3N2) virus for oseltamivir; Clinical impact unknown
	A/Portugal/82/2009	UEOO/NI (4.09) + UMOZ/NI (2.18)					
	A/Portugal/18/2009	UMOO/NI (1.56)	K432E[a]	B → A	No	Global head, antigenic site	Not described
			G454S	Non P → P	No	Global head, antigenic site	Not described
	A/Portugal/03/2011 (suspected case 2)	UEOO/HRI (248.53)	H275Y	B → P	No	Global head, framework residue of active site	Yes—established molecular marker of clinical oseltamivir resistance in N1 viruses
	A/Portugal/10/2011	UMOZ/NI (1.49)	T381N	No	Yes—introduction of potential site	Global head	Not described
HA (numbering without considering signal peptide)	A/Portugal/22/2011	UMOO/NI (1.42)	N44S[b]	No	No	Stalk region	Not described
	A/Portugal/18/2009	UMOO/NI (1.56)	V199I	No	No	HA1 globular head	Not described
	A/Portugal/64/2009	UMOO/NI (1.62)	N276S	No	No	HA1 stalk region (C-terminal)	Not described
	A/Portugal/114/2009	UEOO/NI (2.06)	I286T	Non P → P	No	HA1 stalk region (C-terminal)	Not described
			T288S	No	No	HA1 stalk region (C-terminal)	Not described
	A/Portugal/03/2011	UEOO/HRI (248.53)	N156D	P → A	No	HA1 globular head, antigenic site Sa	Not described
	A/Portugal/07/2011	UMOO/NI (1.56) + UEOZ/NI (2.02)	N129D	P → A	No	HA1 globular head	Not described
			K153E[a,c]	B → A	No	HA1 globular head, antigenic site Sa	Not described
			N156K	P → B	No	HA1 globular head, antigenic site Sa	Not described
			G237R	Non P → B	No	HA1 globular head, antigenic site Ca	Not described

Mutations were identified against A/California/07/2009 vaccine strain sequences.
UEOO, upper extreme outlier oseltamivir; UMOO, upper mild outlier oseltamivir; UEOZ, upper extreme outlier zanamivir; UMOZ, upper mild outlier zanamivir; NI, normal inhibition profile; RI, reduced inhibition profile; HRI, highly reduced inhibition profile; B, basic; A, acidic; NonP, non-polar; P, polar.
[a]Mixed population.
[b]Found in three reference strains with unknown phenotypic profile to NAIs (A/Alborz/5607/2010, A/Baden Württemberg/14/2010 and A/Saint Petersburg/100/2011).
[c]Only found in one reference strain with unknown phenotypic profile to NAIs (A/Baden Württemberg/14/2010).
[d]Based in Maurer-Stroh et al. [2009] and Wei [2010] for NA and in Igarashi et al. [2010] and Yang et al. [2010] for HA.
[e]Based in Nguyen et al. [2012].

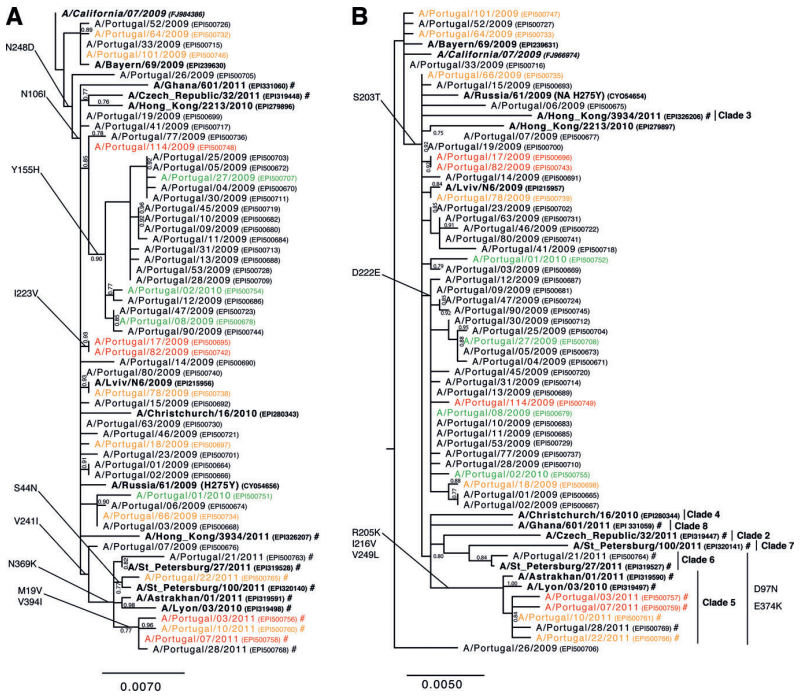

Fig. 3. Phylogenetic analysis of the neuraminidase (**A**) and hemagglutinin (**B**) full-length nucleotide sequences of the 50 A(H1N1)pdm09 virus strains evaluated genotypically, 44 from the pandemic period and 6 from 2010/2011. Nucleotide substitution models GTR+Γ4 (General Time Reversible model with a 4-category gamma distribution for rate variation among sites) and HKY85+I (Hasegawa-Kishono-Yano model with optimized proportion of invariable sites) were used for construction of, respectively, NA and HA maximum-likelihood trees, as determined in jModelTest. aLRT (SH-like) branch support values equal or higher to 0.75 are indicated in the tree. Virus strains are symbol coded according to their phenotypic outlier classification: ### upper extreme outlier; # upper mild outlier; * lower mild outlier (colored web version: virus strains are colour coded according to their phenotypic outlier classification - red: upper extreme outlier; orange: upper mild outlier; green: lower mild outlier; black: non-outlier). When a virus strain was outlier for both NAIs but at different categories, the category associated with a higher IC50 fold-change was used for strain coding. Additionally, all strains from 2010/2011 are coloured in grey (colored web version: all strains from 2010/2011 are marked with the symbol #). Reference virus strains are indicated in bold and uppercase and A/California/07/2009 vaccine strain is additionally in italic. All indicated cluster defining amino acid mutations were identified against vaccine strain sequence. HA established genetic clades are based in [WHO, 2012].

a similar IC_{50} fold-change reduction. Both results support an over all difference between the oseltamivir resistance levels of the H275Y viruses from the two A(H1N1) lineages. In addition to the NA H275Y mutation, genotypic analysis of A/Portugal/03/2011 identified HA N156D as the only other specific mutation of this virus strain. HA mutations at residue 156 (N156D/S/K/T) had been described in A(H1N1)2009 variant as commonly but not exclusively associated with virus propagation in MDCK-SIAT1 cells [WHO, 2011b]. In fact, another polymorphism at this residue (N156K) was observed in this study for one upper mild outlier. HA sequencing from the clinical specimen will be essential for confirmation. Also, residue 156 is located in the antigenic site Sa and therefore N156D mutation could be associated with the key antigenic

role of HA. However, a potential compensatory role for the H275Y fitness deficit cannot be ruled out and could be tested through in vitro replication kinetics assays using wild-type and mutant viruses (without N156D mutation). HA sequencing is important for the study of antiviral drug susceptibility not only due to the potential compensatory role that HA mutations may have but essentially due to the direct impact that HA mutations can have on virus susceptibility. It is well-known that HA and NA proteins work in concert during viral entry and viral release from the cell. Thus, if an HA mutation decreases the virus affinity for binding cell receptors, then it could also reduce the virus NA activity requirement for viral release and consequently reduce the virus susceptibility to any NAI [Zambon and Hayden, 2001].

The identification of the two H275Y oseltamivir-resistant cases is in agreement with the sporadic detection of resistant cases that has been observed worldwide. Oseltamivir-resistant cases have emerged mainly as a result of antiviral drug use and immunosuppressive conditions, both criteria present for suspected case 2 [WHO, 2011a].

The presence of H275Y resistant marker was not detected in the original specimen from clinical suspected resistant case 1. Therefore, the evidence of clinical failure observed can be most probably a result of the high delay between the onset of symptoms and the start of antiviral therapy (11 days).

Other finding of this study was the identification of I223V mutation in the NA sequence of two A(H1N1) pdm09 virus strains exhibiting higher IC_{50} values to oseltamivir (three to four fold-change increase) and, to a lesser extent, to zanamivir (~2-fold-change increase).These virus strains are from two A(H1N1) pdm09 community cases from the 2009 pandemic period. NA I223V mutation was previously associated to a similar decreased susceptibility pattern in N1 viruses but only in one reverse genetic study [Nguyen et al., 2012]. During surveillance activities or clinical studies, this mutation was found in viruses with reduced susceptibility to oseltamivir (fold-change increase >10) in the A(H3N2) and B subtype/type [Nguyen et al., 2012]. The clinical impact of I223V, as of most NAIs reduced susceptibility-conferring mutations, is still unclear. But, mutations at position 223 have previously been associated with clinical NAIs treatment failure [van der Vries et al., 2012]. No epidemiological link was known for the two patients harboring the NA I223V viruses, but the identical NA and HA sequences of the viruses and the temporal and geographic proximity of the cases, indicates a potential relation.

The observed lack of impact of NA Y155H mutation (HRI NAIs phenotype in A(H1N1) seasonal viruses), in the NAIs phenotypic profile of A(H1N1)pdm09 viruses can be explained by structural differences on the NA of A(H1N1) pandemic and seasonal variants. These structural differences could also explain the significantly lower IC_{50} values exhibited by the viruses from the pandemic variant. The absence of the 150-cavity, characteristic of N1 virus, in A(H1N1) pdm09 viruses was initially indicated as the main structural difference [Li et al., 2010]. However, a recent study showed the presence of this cavity in all N1 and, surprisingly, in N2 viruses, raising new questions about NA structures [Amaro et al., 2011].

No genetic mechanism was found for the IC_{50} minor increase (1.4- to 2.1-fold-change) observed for most of oseltamivir and zanamivir upper outlier virus strains. No shared mutation was identified and approximately half of these virus strains lacked any specific mutations at NA or HA sequences. Additionally, the majority of the specific mutations identified probably derived from the key antigenic role of these two glycoproteins, given their location in antigenic sites or by creating potential N-glycosylation sites that may prevent accessibility and recognition of antigenic sites by antibodies [Vigerust et al., 2007]. Other specific mutations, positioned in the stalk region of the protein, are most likely not related with antiviral susceptibility. Whole genome sequencing of upper outlier strains is being performed in order to find if mutations located in the other genome segments can be contributing for the IC_{50} minor increases observed. The understanding of these minor increases is one of the major current challenges in influenza antiviral drug susceptibility evaluation.

A(H1N1)pdm09 viruses were more susceptible to zanamivir than to oseltamivir. This drug-specific variation is characteristic of N1 viruses and results from differences in the intrinsic chemical properties of the two NAIs [Ferraris and Lina, 2008]. The chemical similarity that zanamivir exhibits to the natural substrate of NA (sialic acid) could explain the different phenotypic results obtained for this drug [van der Vries et al., 2012]. This includes the lack of significant differences between the IC_{50} values from the two time periods analyzed and the lower impact of NA I223V mutation on virus susceptibility (lower IC_{50} fold-change). Regarding oseltamivir, the two NA-specific mutations of 2010/2011 virus strains —N369K and V241I, may have contributed for the significantly higher IC_{50} values obtained during that season. Computational analysis showed that both mutations improve NA stability of H275Y oseltamivir-resistant virus and experimental studies showed that N369K increases NA surface expression and activity of this resistant variant [Hurt et al., 2012]. However, the unequal size of the two time period datasets (N = 116 for the pandemic period and N = 26 for 2010/2011) may also be contributing for this IC_{50} temporal difference.

The results obtained in this study may contribute to a better understanding of the currently challenging phenotype–genotype relationship. In addition, all national data presented here may contribute to the global assessment of A(H1N1)pdm09 virus susceptibility profile and baseline level to NAIs.

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APPENDIX B

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Genomic signatures and antiviral drug susceptibility profile of A(H1N1)pdm09

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ABSTRACT

Background: Genetic changes in influenza surface and internal genes can alter viral fitness and virulence. Mutation trend analysis and antiviral drug susceptibility profiling of A(H1N1)pdm09 viruses is essential for risk assessment of emergent strains and disease management.
Objective: To profile genomic signatures and antiviral drug resistance of A(H1N1)pdm09 viruses and to discuss the potential role of mutated residues in human host adaptation and virulence.
Study design: A(H1N1)pdm09 viruses circulating in Portugal during pandemic and post-pandemic periods and 2009/2010 season. Viruses were isolated in MDCK-SIAT1 cell culture and subjected to mutation analysis of surface and internal proteins, and to antiviral drug susceptibility profiling.
Results: The A(H1N1)pdm09 strains circulating during the epidemic period in Portugal were resistant to amantadine. The majority of the strains were found to be susceptible to oseltamivir and zanamivir, with five outliers to neuraminidase inhibitors (NAIs) identified. Specific mutation patterns were detected within the functional domains of internal proteins PB2, PB1, PA, NP, NS1, M1 and NS2/NEP, which were common to all isolates and also some cluster-specific.
Discussion: Modification of viral genome transcription, replication and apoptosis kinetics, changes in antigenicity and antiviral drug susceptibility are known determinants of virulence. We report several point mutations with putative roles in viral fitness and virulence, and discuss their potential to result in more virulent phenotypes. Monitoring of specific mutations and genetic patterns in influenza viral genes is essential for risk assessing emergent strains, disease epidemiology and public health implications.

1. Background

The emergence of virulent influenza phenotypes is a consequence of genetic changes altering the function of individual proteins or their functional compatibility.

Genetic changes in the viral surface proteins can impact on virus binding, entry, assembly, release, induction of the host's immune response and antiviral drug resistance.

Within the internal proteins, genetic changes can impact on virulence and infectivity if they alter the ability of the virus to replicate or induce cellular apoptosis.[1] The viral replication process is multigenic and interactive, involving the replication complex and the structural and functionally associated M1 and NS2/NEP proteins.[1,2] The gene segments coding for the proteins involved in replication, code for additional proteins associated with the induction of cellular apoptosis during viral infection. The (+1)ORF of PB1 encodes PB1-F2 with pro-apoptotic function in the host cells and the NS gene encodes NS1 protein associated with the activation of anti-apoptotic mechanisms, permitting viral replication to occur.[3–5] The cassette of internal genes therefore controls the major processes by which viral fitness is determined: replication and apoptosis. An increased replication rate or stimulation of anti-apoptotic mechanisms, produces higher viral loads that can overcome host immune response, enhance infectivity and result in a more severe and transmissible disease. The most severe epidemiological situations caused by seasonal influenza viruses occurred in 1947 and 1951 as a consequence of genetic changes within internal proteins involved in replication of A(H1N1) viruses. The strains spread worldwide and caused exceptionally high mortality.[6]

Monitoring evolutionary trends in the genes encoding influenza virus internal proteins, and profiling antigenic proteins and antiviral drug susceptibility, are therefore essential to risk assessing influenza virulence, disease epidemiology and public health implications.

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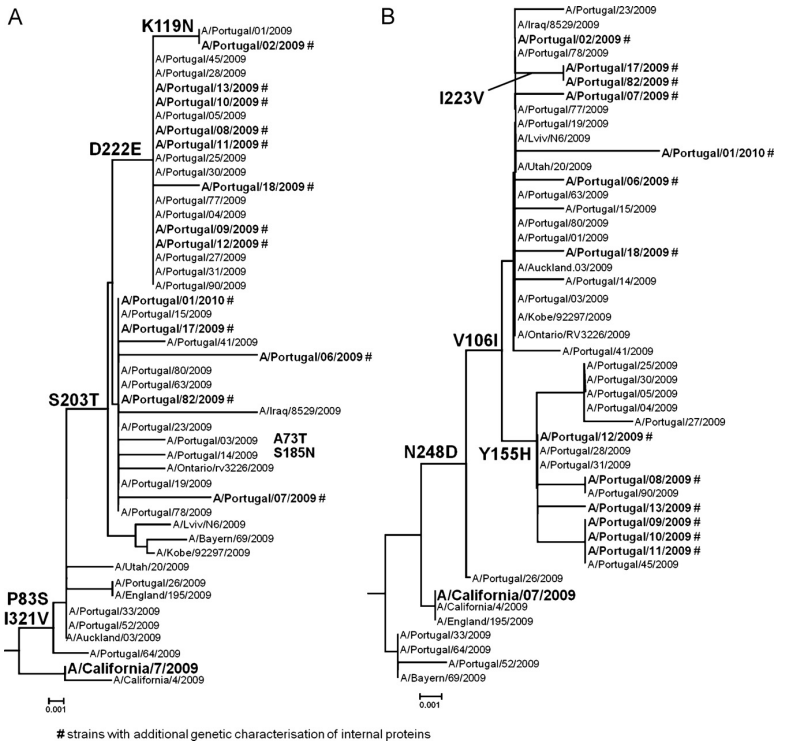

Fig. 1. Phylogenetic analysis of the aminoacid sequences of HA1 subunit of hemagglutinin (1A) and neuraminidase (1B) genes of influenza A(H1N1)pdm09 strains circulating in Portugal.

2. Objectives

With the aim of risk assessing emergent strains, the main objectives of the study were to profile genomic signatures and antiviral drug susceptibility of A(H1N1)pdm09 viruses and discuss the potential putative role of mutated residues in human host adaptation and virulence.

3. Study design

A total of 110 specimens from laboratory-confirmed cases of pandemic influenza were collected in Portugal from July 2009 to January 2010. Strains were isolated in MDCK-SIAT1 cell culture. Antigenic surface glycoproteins and M1 and M2 proteins were genetically characterized and antiviral drug susceptibility was profiled. Genetic characterization of internal proteins was performed on viruses isolated from subsets of pregnant women, outliers to NAIs and a 14 years old deceased patient with no risk factors. Cycle sequencing was performed based on adaptation of a protocol from CDC, recommended by WHO.[7] Genetic mutation analysis and phylogenetic analysis were performed with LasergeneV.4.05-DNASTAR and MEGA4.0, with comparison to sequences of the A(H1N1)pdm09 2010/2011 vaccine strain (A/California/7/2009) and published reference A(H1N1)pdm09 strains circulating worldwide within pandemic and post-pandemic periods. Phenotypic evaluation of antiviral drug susceptibility was performed by fluorescent assay with MUNANA substrate as previously described.[8] Minor and major phenotypic outliers were identified through the establishment of lower and upper IC_{50} cut offs values.[8] Genotypic evaluation of antiviral drug susceptibility to NAIs and to amantadine was performed by analysing NA and M2 protein sequences, respectively. In the M2 sequence the 5 well-defined molecular markers of resistance to amantadine were analysed: L26F/I; V27A/D; A30T; S31N and G34E.

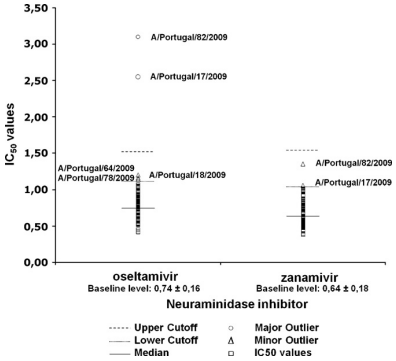

Fig. 2. Phenotypic evaluation of A(H1N1)pdm09 virus susceptibility to NAIs by fluorescence assay.

4. Results

Virus isolation was performed for all 110 A(H1N1)pdm09 cases. Of these, 37 were genetically characterized for antigenic glycoproteins and for M protein. Antiviral drug susceptibility profile was phenotypically evaluated for 103 of the 110 isolates. Ninety-eight were further sequenced for genotypic evaluation of NAIs susceptibility and 96 for amantadine susceptibility. Genetic characterization of internal proteins PB2, PB1, PB1-F2, PA, NP, NS1 and NS2/NEP was performed for 13 of the 37 isolates, including viruses isolated from cases in the subsets described above.

Within the HA1 domain of the HA, all 37 strains were shown to contain the mutations P83S and I321V (Fig. 1A). Thirty-three of the 37 strains had the substitution S203T, and 19 also contained D222E. Additional point mutations were observed, including A73T and S185N in individual strains, which are located in the putative antigenic sites Cb and Sb, respectively. The substitution K119N which results in the creation of an N–X–S motif, was observed in 2 strains. In the NA gene, 34 of the 37 strains analysed contained N248D and V106I substitutions (Fig. 1B). Fifteen strains had Y155H and two contained I223V in the NA gene.

The genetic characterization of MP of 37 strains identified M128L, E201D and M203I substitutions in individual isolates compared to the A(H1N1)pdm09 2010/2011 vaccine strain MP sequence, located within the M1 coding region.

Phenotypic evaluation of NAIs susceptibility revealed three minor and two major outliers to oseltamivir, as shown in Fig. 2. The three minor outliers exhibited reduced susceptibility of approximately two-fold and the two major outliers of approximately 3- and 4-fold, compared to baseline level. The two major outliers were also found to be minor outliers to zanamivir (MjOOsel/MnOZana), with reduced susceptibility of approximately two-fold compared to baseline level. In the NA sequence of these two MjOOselt/MnOZana strains, the mutation I223V was observed. Genotypic evaluation of susceptibility to amantadine revealed that all 96 strains analysed had an aspargine (N) at position 31 in the M2 coding region.

The genetic profile of the internal proteins of 13 A(H1N1)pdm09 isolates analysed revealed substitutions P224S in PA, V100I and L122Q in NP and I123V in NS1, which were common to all 13 isolates (highlighted in bold and #, Fig. 1), as shown in Table 1. PB1-F2 was present in the truncated non-functional form of 11 amino acids.

Table 1
Non-synonymous mutations detected per gene/protein. Strains are ordered according to epidemiologic week of beginning of symptoms.

Strain	Epidemiological week	Gene/protein						NS2/NEP	Observations
		PB2	PB1	PA	NP	NS1			
A/Portugal/17/2009	30/2009	V480I	–	P224S	V100I; L122Q	I123V		–	MjO Osel; MnO Zana
A/Portugal/82/2009	32/2009	V480I; D680E	–	P224S	V100I; L122Q; K400R	I123V		–	MjO Osel; MnO Zana
A/Portugal/2/2009	34/2009	–	–	P224S	V100I; L122Q	I123V; F103C		–	n.o.
A/Portugal/7/2009	34/2009	–	S633G	P224S	V100I; L122Q	I123V		–	Pregnancy
A/Portugal/8/2009	39/2009	R54K; I463M	–	P224S; D347N; M561I	V100I; L122Q	I123V		–	Pregnancy
A/Portugal/13/2009	39/2009	R54K	S384L	P224S; D347N; C95G; N409S	V100I; L122Q	I123V		S93G	Pregnancy
A/Portugal/9/2009	43/2009	R54K; I354V	S384L	P224S; D347N	V100I; L122Q	I123V; A155V; R211K		D54N	Pregnancy
A/Portugal/10/2009	44/2009	R54K	S384L	P224S; D347N	V100I; L122Q	I123V; A155V; R211K		D54N; G22E	Pregnancy
A/Portugal/11/2009	44/2009	R54K; E249G	S384L	P224S; D347N	V100I; L122Q	I123V; A155V; R211K		D54N	Pregnancy
A/Portugal/18/2009	45/2009	–	–	P224S; S296N	V100I; L122Q	I123V; P114H		–	MnO Osel; ICU
A/Portugal/12/2009	49/2009	R54K	–	P224S; S364N; N614S	V100I; L122Q	I123V		–	Deceased
A/Portugal/6/2009	50/2009	E191K	–	P224S; Q193K; P400L	V100I; L122Q	I123V		–	Children
A/Portugal/1/2010	04/2010	K340N	S678N	P224S; I94L; N675H	V100I; L122Q; V217I; V313I	I123V; A155V		–	Pregnancy

MjO, major outlier; MnO, minor outlier; Osel, oseltamivir; Zana, zanamivir; ICU, intensive care unit; n.o., no observations.

Viruses isolated from the subset of pregnant women, were observed to have R54K substitution in PB2 in 6 cases, 5 of which also contained D347N in PA. Of these, 4 also contained S384L in PB1 and a small cluster of 3 isolates was characterized by the additional substitutions A155V and R211K in NS1, and D54N in NS2/NEP.

The mutation V480I was found only in the PB2 sequence of the two strains exhibiting a reduced susceptibility profile to both NAIs (MjOOselt/MnOZana).

5. Discussion

A(H1N1)pdm09 viruses characterized by the HA substitution S203T and both N248D and V106I in NA, were found to be the dominant circulating strain throughout the epidemic period (2009–2010) in Portugal. Previous studies have also reported dominant circulation of this strain worldwide and suggested this is a consequence of enhanced viral fitness.[9,10]

Within HA1, there was no evidence of evolutionary trends, in agreement with the genetic and antigenic homogeneity of A(H1N1)pdm09.[11] The location of amino acid substitutions observed in HA1 however, suggests these could result in phenotypic changes; the D222E substitution is located within a loop of the receptor-binding site, A73T and S185N within the putative antigenic sites Cb and Sb, respectively, and K119N has been reported to result in creation of a potential N-glycosylation site.[9,12] Correlation of these mutations with particular phenotypes, binding specificity or clinical outcome of infection however, needs further investigation.

In the internal genes, the V100I and L122Q substitutions observed in NP, I123V in NS1 and P224S in PA characterize the genetic profile of all isolates analysed, reflecting the dominant circulation of this A(H1N1)pdm09 genetic variant in Portugal within the period analysed. Residues 100 and 122 in NP are located within the body domain of the protein and are thought to be involved in PB2 and PB1–NP interaction, which is crucial for RNA replication. The amino acid substitution V100I was associated with the raising of the pandemic alert from phase 4 to 6, and is a human to avian signature change known to translate into enhanced viral fitness probably by increasing viral transmissibility or infectivity.[10,13] I123V in NS1 is located in the effector domain, a functional region regulating cellular apoptosis, and therefore suspected to play a role in adaptation to the human host and to increase virulence.[10] P224S in PA is located in the N-terminal domain, which has endonuclease activity. Although no phenotypic outcome for this mutation has been established to date, endonuclease activity is critical for initiating transcription and therefore, genetic changes in this region might be expected to alter replication kinetics.[14] Although the phenotypic outcome of L122Q has not been established, this mutation has been previously reported as a virulence factor in H5N1 by greatly increasing replication.[15]

In the viruses from pregnant women, in addition to the mutations common to all isolates analysed, a unique profile of 6 genetic signatures in the functional domains of internal proteins defined a specific cluster. Considering their genetic location, they present a high potential for translating into phenotypic changes in fitness and virulence. Both R54K in PB2 and D347N in PA are located within functional domains for binding PB1.[7,18] Since the function of the polymerase proteins depends on the formation of a heterodimer for optimal viral RNA replication and transcription, these substitutions together with P224S in PA, may have impacted on polymerase subunit binding to PB1 and have had an unpredictable effect on replication in these strains.[16,17] Within NS1, the cluster is defined by A155V and R211K substitutions, both located within the C-terminal of the effector domain, as is I123V, which has previously been found to regulate cellular apoptosis.[5,18] Replication and apoptosis are the major mechanisms that define viral fitness. The occurrence of different point mutations as occurred in strains in this cluster, could be compensatory or have had a cumulative effect leading to a different phenotype. These mutations will continue to be monitored in non-pregnant patients to evaluate whether they are subset exclusive, resulting from enhanced function of individual proteins or increasing functional compatibility of proteins.

The virus isolate from the deceased patient contained a distinctive set of mutations which could have contributed to this more severe clinical outcome. In addition to N248D and V106I in NA, S203T in HA, V100I in NP and I123V in NS1, this isolate contained L122Q in NP, S364N and N614S in PA, and R54K in PB2, all located in PB1 binding domains of the respective proteins.[17] A direct association of PB1 binding to the other polymerase subunits and to NP with a more virulent phenotype has not been established to date. However, the formation of PB2–PB1–PA heterodimer and the binding of PB2 and PB1–NP into ribonucleoproteins both determine vRNA transcription and cRNA synthesis for genome replication and therefore, have the potential to greatly affect the viral replication cycle.[17]

Within the subset of outliers to NAIs, the two MjOOsel/MnOZana were defined by I223V in NA and V480I in PB2. Amino acid substitutions at position 223 in NA have previously been shown to affect virus susceptibility to both oseltamivir and zanamivir, in recent A(H1N1)pdm09 and seasonal viruses.[19,20] Residue 223 is located in the framework of the NA active site, and thus interacts with the catalytic residues to which antiviral drugs bind.[21] The contribution of V480I in PB2 to the reduced susceptibility profile observed is unclear. In addition, no phenotypic impact was observed in A(H1N1)pdm09 viruses containing NA mutation Y155H, previously observed in seasonal A(H1N1) viruses resistant to both NAIs.[22]

Correlating specific mutations with virulent phenotypes in the human host is not straightforward. Animal models differ in their immune response to infection and multiple other factors interfere with clinical and epidemiology outcomes of infection. Also, the genetic background of specific mutations is divergent among influenza strains and can have different phenotypic outcomes. Several mutations have however, been recognized as virulence markers. Although some mechanisms remain unclear, the modification of genome transcription, replication, apoptosis and antigenicity are determinants of virulence. Additionally to known molecular markers of virulence, we have placed selected residues within functional domains of internal proteins that may enhance viral fitness or increase virulence under genomic surveillance.

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Competing interest

None declared.

Ethical approval

No human subjects were used in this research study. Specimens were accessed through anonymous banks, reviewed and exempt for specific issue document from the Ethic Commission of the National Institute of Health, Lisbon, Portugal.

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APPENDIX C

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Genetic and antiviral drug susceptibility profiles of pandemic A(H1N1)v influenza virus circulating in Portugal

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Abstract

Global A(H1N1)2009 genetic characterization, molecular evolution dynamics, antiviral susceptibility profiles, and inference of public health implications require nation and region wide systematic analysis of circulating virus. In this study we analysed the genetic and antiviral drug susceptibility profiles of pandemic A(H1N1)2009 influenza virus circulating in Portugal. Genetic profile analysis was performed in 37 isolates to the hemagglutinin (HA), neuraminidase (NA) and MP genes, and in six of these isolates the PB1, PB2, PA, NP and NS genes were also analysed. Antiviral drug susceptibility profile was analysed for 96 isolates, phenotypically and genotypically to neuraminidase inhibitors (NAI) and genotypically to amantadine. The point mutations identified in HA, NA, and MP genes of different strains do not seem to evidence an evolutionary trend. This is in agreement with the genetic and antigenic homogeneity that has being described for A(H1N1) 2009 virus. All analysed strains were found to be resistant to amantadine, and five of these strains exhibited a reduced susceptibility profile to NAI, three only for oseltamivir and two for both inhibitors.

Introduction

In mid-April 2009 a novel variant of A(H1N1) influenza virus began to spread rapidly throughout the world, causing the first pandemic of the 21st century. The majority of the cases associated with this new virus show to be mild, but severe and fatal cases have been reported. Molecular markers associated with severity have already been identified, as is the case of the mutation D222G.[1] Resistant viruses to antiviral drugs have also been identified, highlighting the importance of rapid determination of the antiviral drug profile.

Global A(H1N1) 2009 genetic characterization, molecular evolution dynamics, antiviral susceptibility profiles, and inference of public health implications require nation and region wide systematic analysis of circulating virus.

The objective of this ongoing research study was, primarily, to thoroughly characterize the genetic profile and evolution of the emergent influenza A(H1N1) 2009 virus circulating in Portugal and its phenotypic expression on antiviral drugs susceptibility.

Materials and methods

The cases considered in this study were obtained from the community and from two collaborating hospitals in Lisbon – a reference hospital for adults (Hospital de Curry Cabral) and a reference hospital for children (Hospital Dona Estefânia).

The CDC real-time PCR protocol, recommended by World Health Organization (WHO), was the method used to confirmed all influenza A(H1N1) 2009 cases. From a total of 577 A(H1N1) 2009 positive cases diagnosed and confirmed, 163 were selected for this study, taking in consideration that they should cover the period of epidemic activity in Portugal and include cases from persons belonging to risk groups and cases associated with more severe clinical features. Ninety-six A(H1N1) 2009 strains were isolated in MDCK-SIAT1 cells, from combined naso-oropharyngeal swabs.

For the evaluation of the genetic profile of A(H1N1) 2009 virus circulating in Portugal, 37 of the 96 isolates were characterized by genetic analysis of the HA, NA, and MP genes. The remaining five gene segments (PB1, PB2, PA, NS, and NP) were also sequenced for six of this 37 isolates. Briefly, sequencing was performed according to the protocol developed by CDC and recommended by WHO,[2] using BigDye Terminator V.1.1 technology. Nucleotide sequences were determined in a DNA automatic sequencer ABI PRISM 3130XL Genetic Analyzer. For each genomic segment, genetic analysis was performed with Lasergene V.4.05 software (DNASTAR Inc, USA) using an average of 4–6 overlapping readings, including sense and antisense, for precise nucleotide and amino acid sequence determination. Genetic mutation and phylogenetic analysis were performed by neighbor-joining method, using MEGA4.0 software, against published sequences from the vaccine strain (A/California/7/2009) and from selected A(H1N1) 2009 strains available on GISAID EpiFlu Database. All mutations were identified with reference to the vaccine strain genome sequence.

Antiviral drug susceptibility profile of A(H1N1) 2009 influenza virus circulating in Portugal was evaluated both phenotypically and genotypically for NAIs and genotypically for amantadine.

Phenotypic evaluation to NAIs, oseltamivir and zanamivir, was performed for all 96 isolates by IC_{50} determination through MUNANA fluorescence assays.[3] Genotypic evaluation was performed by searching for mutations associated with resistance to NAIs in all 37 NA gene sequences. Amantadine susceptibility profile was performed for all 96 isolates by searching on M2 sequence for the 5 molecular markers associated with resistance to this antiviral drug (L26F/I; V27A/D; A30T; S31N; G34E).

Results

Genetic characterisation of the HA1 subunit of HA reveals point mutations in different strains. All 37 analysed strains present P83S and I321V mutations, which distinguish them from the vaccine strain (Figure 1A). Thirty-three of the 37 sequenced strains group in the S203T branch. This mutation is referred in the literature as being associated with the putative antigenic site Ca.[4] Most of these strains (19) further subgroup in the D222E branch, this mutation being associated with one loop of the receptor-binding site.[1]

From the early to the late epidemic period, an increased circulation of virus carrying the mutation S203T was observed. This is in agreement with the association between this mutation and an enhanced viral fitness that is described in the literature.[5] Additional mutations were also observed in a small number of virus, of which we highlight: (i) mutation A73T in A/Portugal/03/2009 and mutation S185N in A/Portugal/14/2009, associated with the putative antigenic sites Cb and Sb respectively,[4] and (ii) mutation K119N in A/Portugal/01/2009 and A/Portugal/02/2009 that can result in a new N-glycosilation site, as it originates an N-X-S motif in the aminoacid sequence.

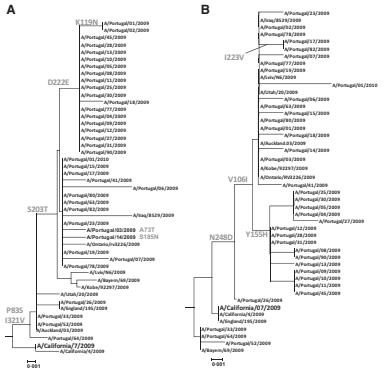

Figure 1. Phylogenetic tree of the amino acid sequences of HA1 subunit of hemagglutinin (A) and neuraminidase (B) genes of Influenza A(H1N1)2009 strains.

Regarding the genetic characterisation of NA, the majority of strains analysed (34 of 37) presents the mutations N248D and V106I (Figure 1B). As mutation S203T in HA gene, these two NA mutations are described in the literature as associated with enhanced viral fitness.[5] The few strains not carrying these mutations have circulated in the beginning of the epidemic period. Fifteen of the 37 analysed strains further subgroup in Y155H branch. Additionally, mutation I223V was identified in two strains.

The analysis of the complete MP gene of 37 strains revealed three mutations in the M1 protein (M128L in A/Portugal/45/2009, E201D in A/Portugal/26/2009, and M203I in A/Portugal/77/2009) and three in the M2 protein (P10H in A/Portugal/26/2009, R18K in A/Portugal/03/2009, and E66K in A/Portugal/28/2009). However, no reference to these mutations was found in the literature.

For the remaining gene segments available for the six analysed strains, the observations include: (i) no previously described virulence markers in PB2, PB1-F2, and NS1 were detected; (ii) PB1-F2 protein is present in the truncated form of 11 amino acids; (iii) the presence of mutations I123V and L122Q in NS1 and V100I in NP; (iv) the described association of mutation I123V in NS1 and V100I in NP genes with viral fitness.[5]

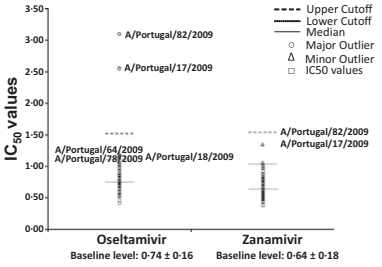

Figure 2. IC_{50} values obtained by fluorescence assay for influenza A(H1N1)2009.

Phenotypic evaluation of NAIs susceptibility revealed the existence of three minor and two major outliers to oseltamivir (Figure 2). The two minor outliers exhibited a reduction of approximately twofold in the susceptibility to this antiviral drug, comparing to the baseline level, while the reduction exhibited by the two major outliers was of approximately three- and fourfold.

Regarding zanamivir, two minor outliers were identified with a reduction of approximately twofold in the susceptibility, compared to the baseline level. These two minor outliers (A/Portugal/17/2009 and A/Portugal/82/2009) correspond to the two major outliers identified for oseltamivir.

Genetic analysis revealed the presence of the mutation I223V in the NA sequence of these two strains. The contribution of this mutation for the profile of reduced susceptibility identified for both NAIs is not known, but a mutation in the same NA position (I223R) has been referred to as being associated with a reduction in NAIs susceptibility.[6] Full genome sequence analysis of these strains shows that both strains also present the V480I mutation in PB2 gene. However, no association of this mutation with antiviral drug susceptibility is referred in the literature.

Concerning genetic evaluation of susceptibility to amantadine, all 96 analysed strains present a Serine in position 31, which is a molecular marker of resistance to M2 inhibitors.

Discussion

These preliminary results allow us to discuss several points. However, the additional data that is being obtained through this ongoing study will be essential for a more complete analysis. For example, more information is needed to determine if the mutations found alter the biology and the fitness of the virus or if there are associated with an increased prevalence of the virus.

The majority of the mutations identified in HA1 subunit have been detected in A(H1N1)2009 strains distributed throughout the epidemic curve, not evidencing a specific evolutionary trend. This is in agreement with the genetic and antigenic homogeneity that has being described for A(H1N1)2009 virus.[7]

The occurrence of mutations in the position 222 of the HA1 subunit of A(H1N1)2009 virus have been described. However, more studies are needed to clarify the outcome of these mutations, as for example in patients with severe complications. It could also be relevant to investigate the presence of single and mixed variants in viruses and in clinical specimens and the possibility of these mutations affecting the binding specificity.

Regarding the susceptibility of A(H1N1)2009 pandemic viruses to antiviral drugs, all analysed strains were found to be resistant to amantadine. This resistant profile was not unexpected since the MP gene from this new variant had originated in the Eurasian swine lineage, which is characterised by being resistant to this antiviral drug.[8]

The majority of the A(H1N1)2009 strains analysed revealed to be susceptible to both NAIs, with only five strains exhibiting a profile of reduced susceptibility, three to oseltamivir and two to both NAIs. For these last two, the presence of the I223V mutation in the NA sequence could explain the reduction observed, but a more complete analysis is needed to confirm this.

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A(H1N1)2009 pandemic in France: epidemiological features based on virological surveillance

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Abstract

The French national pandemic plan includes an early containment phase followed by a limitation phase. The efficacy of such a plan depends on pre-existing surveillance and laboratory networks. The GROG community surveillance network and the hospital lab networks organized by the two French NICs carried out the virological monitoring of the A(H1N1)2009 pandemic. The NICs set up and distributed the RT-PCR tools to the lab networks early May 2009. During the containment phase, all suspected and virologically confirmed cases were hospitalized and declared to InVS. During the limitation phase, the clustered cases were monitored, and GROG swabs were collected by practitioners in general population. During the pandemic, the NICs carried out additional testing for the monitoring of antiviral resistance and of genetic changes involved in virus adaptation (PB2) and virulence (HA). The first imported A(H1N1)2009 influenza cases were detected at the end of April 2009. Local transmission was observed at the end of May. Clusters were observed in schools in June and in summer camps during summer. Sporadic cases were reported up until October when the pandemic wave started. One single 10 week-long pandemic wave was observed between mid-October and the end of December. Overall, 103 352 samples have been tested with 24 279 positives. The weekly positive rate ranged from 0% to 48% with a peak week 48 (3877 positives). Phylogenetic and antigenic analyses did not show any emerging genetic or antigenic variants. Eight cases had a D222G mutation in the HA. Eleven cases had an oseltamivir-resistant virus (H275Y); one harboured a reduced sensitivity to zanamivir (additional I223R mutation). All but one resistant virus were detected in treated immunocompromised patients. According to the profile of hospitalized cases, A(H1N1)2009 was more virulent than seasonal viruses. Even if the mortality was limited (312 cases), the age distribution of the deceased patients was different as compared to seasonal influenza (75% mortality in <65 years of age). The virological monitoring of the pandemic was achieved by the preexisting seasonal influenza networks.

Introduction

The French national pandemic plan, prepared in response to the A(H5N1) pandemic threat, has been implemented in France since 2003 with updates in 2006 and 2008. This plan describes the response to a pandemic with an early containment phase to delay the virus circulation in the population, followed by a limitation phase to contain as much as possible virus circulation and avoid excess hospitalisations and deaths. These phases should use mitigation measures, antivirals, and vaccination, if available.

The efficacy of such plan depends on pre-existing influenza surveillance and laboratory networks. In France, the community surveillance is carried through the GROG surveillance network. In addition, surveillance is also carried out in hospitals by the RENAL network. This RENAL network is divided in two sub-networks: the so-called H5-labs network, activated during the containment phase and the Extended RENAL Lab network activated in the limitation

APPENDIX D

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Antiviral drug profile of seasonal influenza viruses circulating in Portugal from 2004/2005 to 2008/2009 winter seasons

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ABSTRACT

A research project on antiviral drug resistance of influenza viruses circulating in Portugal has been carried out since 2007. Here, the first results obtained regarding the evaluation of susceptibility to amantadine and oseltamivir are presented. Information about antiviral prescription and exposure was available through the National Influenza Surveillance Programme. Amantadine susceptibility was evaluated by pyrosequencing for known resistance markers on 178 influenza A strains from 2004/2005 to 2006/2007. Susceptibility to oseltamivir was evaluated by 50% inhibitory concentration determination on 340 virus strains from 2004/2005 to 2008/2009, 134 of which were further analyzed by sequencing of the neuraminidase gene. This study revealed that influenza antiviral drugs were rarely prescribed at national level. Resistance to amantadine was observed on only A(H3N2) strain isolated during 2005/2006 and on 38 (74.5%) of the 51 A(H3N2) strains from 2006/2007, all carrying the mutation S31N in their M2 sequence. Oseltamivir resistance was observed in 6 (20.7%) of the 29 A(H1N1) strains from 2007/2008 and in all strains from 2008/2009, which exhibited extremely high IC_{50} values and carrying the mutation H275Y in their neuraminidase sequence. The national data generated and analyzed in this study may contribute to increase the knowledge on influenza antiviral drug resistance which is a problem of global concern.

1. Introduction

Two classes of antiviral drugs are currently licensed for the prevention and treatment of seasonal, zoonotic and pandemic influenza: (1) M2 inhibitors or adamantanes, including amantadine and rimantadine, and; (2) neuraminidase inhibitors (NAIs), such as oseltamivir and zanamivir (Crusat and de Jong, 2007). In Portugal, only rimantadine is not licensed for clinical use. Amantadine was introduced into clinical practice in 1973, followed by zanamivir in 1999 and oseltamivir in 2002, all 3 requiring medical prescription (information available at www.infarmed.pt).

The introduction of these antiviral drugs into clinical practice raised public health concerns regarding the potential emergence of resistance and its impact on the clinical effectiveness of the drugs. These concerns led to national and global surveillance activities as well as to specific and detailed research studies on influenza antiviral drug resistance, particularly after the introduction of NAIs.

Resistance to M2 inhibitors has been observed with a high frequency not only in the clinic but also in the community settings, limiting their use on influenza prevention and control. More specifically, resistance was identified on 30–80% of hospitalized patients, within 48–72 h after onset of antiviral therapy (Democratis et al., 2006). In addition, a high increase in the frequency of influenza A(H3N2) virus resistant to M2 inhibitors started to be identified at global level during the 2002/2003 winter season, with the most recent data from 2008/2009 revealing a 100% worldwide frequency of resistance (Bright et al., 2005; WHO, 2009a). High frequencies of resistance to these inhibitors have also been identified for A(H5N1) avian influenza viruses, particularly those from clade 1 and approximately 80% from subclade 2.1. A 100% frequency of resistance was also observed for the recently emerged A(H1N1) swine 2009 pandemic virus (Crusat and de Jong, 2007; Hayden, 2007; WHO, 2009b). Resistance to M2 inhibitors has also been identified for seasonal A(H1N1) influenza virus but on a minor extent and at variable levels, not only between seasons but also between countries. This variability is associated with the co-circulation of 2 hemagglutinin (HA) A(H1N1) lineages, 1 resistant to M2 inhibitors (HA subclade 2C)—represented by A/Hong Kong/2652/2006, and 1 susceptible to this antiviral drug class (HA subclade 2B)—represented by A/Brisbane/59/2007 (Barr et al., 2008; Cheng et al., 2009). However, A/Brisbane/59/2007-like virus have been spreading at global level since they emerged, during 2007/2008, replacing A/Hong Kong/2652/2006-like virus (see interim reports from WHO Influenza Centre, London, available at http://www.nimr.mrc.ac.uk/wic/report/). The most recent data from 2008/2009 revealed that only 2% of the seasonal A(H1N1) virus analysed worldwide belong to the M2 resistant A/Hong Kong lineage (WHO, 2009a).

Regarding the emergence of resistance to NAIs, different situations have been observed for the 2 antiviral drugs available. Resistance to zanamivir has been rarely detected, with the 2 cases identified following treatment of immunocompromised patients being the most notable (Lackenby et al., 2008a). This rare detection can be associated with the high similarity that zanamivir exhibits, at structural level, to the natural substrate of neuraminidase (NA) (Moscona, 2009). However, it is important to consider that zanamivir has been rarely used in the management of influenza, which could also explain the situation observed. Resistance to oseltamivir was, until 2007, detected at a low frequency and mainly following treatment (in 0.32% of the adults and 4.1% of the children under therapy) (Aoki et al., 2007). Two high frequencies of 18% and 16.3% were, however, observed in 2 Japanese clinical trials carried out in children infected with A(H3N2) and A(H1N1) influenza viruses, respectively (Kiso et al., 2004; Ward et al., 2005). During the 2007/2008 winter season, the emergence and widespread circulation of seasonal A(H1N1) influenza virus resistant to oseltamivir, in the absence of drug selective pressure, were identified at global level (ECDC, 2008; Lackenby et al., 2008a). This resistant virus persisted and increased in frequency during 2008/2009, representing 96% of the seasonal A(H1N1) virus that circulated worldwide during that winter season (WHO, 2009a). Sporadic cases of resistance to oseltamivir have been identified for A(H5N1) avian influenza virus, with a total of 5 cases reported (3 in Vietnam and 2 in Egypt), and on the recently emerged A(H1N1) swine 2009 pandemic virus, with a total of 39 cases reported as of 23th October 2009. The majority of these sporadic cases were associated with antiviral drug use (de Jong et al., 2005; Le et al., 2005; Saad et al., 2007; WHO, 2009c,d).

Since 2007, a research project has being carried out in Portugal aiming at the evaluation and study of influenza antiviral drug resistance. With the data that has already been obtained, and with data that is expected to be gathered in the course of this project, it may become possible to contribute to the information that is continually being generated at international level, some of which is summarized above, and to advances in the knowledge on this specific area of research.

In this study, the first results of evaluation of susceptibility to oseltamivir and amantadine of seasonal influenza A and B viruses, circulating in Portugal from the 2004/2005 to the 2008/2009 winter seasons, obtained through the national research project mentioned above are reported and discussed.

2. Materials and methods

2.1. Influenza virus strains analysed

The influenza virus strains analysed in this study were isolated from nasopharyngeal swabs collected from influenza-like illness patients who consulted with a Sentinel Medical Practitioner or attended an Emergency Unit participating in the National Influenza Surveillance Programme. The respiratory specimens were accompanied by a notification form containing epidemiological and clinical information related to the patient and, since 2005/2006, information regarding prescription and exposure (either by direct use or contact with a patient on therapy) of patients to influenza antiviral drugs before specimen collection. Every time a notification form indicated drug prescription and the presence of influenza virus was detected on the swab, a second respiratory specimen from the patient was requested, collected at the end of antiviral therapy (4th or 5th day).

Viral isolation was performed in MDCK or MDCK-SIAT1 cells, according to the WHO Manual on Animal Influenza Diagnosis and Surveillance (2002). Antigenic characterization of viral isolates was performed by (1) hemagglutination inhibition assays for determination of HA type or subtype, following the protocol included in the WHO Influenza Reagent Kit for Identification of Influenza Isolates, and; (2) TaqMan real-time PCR for determination of neuraminidase (NA) influenza A subtype, using a modified version of the protocol described by Schweiger et al. (2000).

Susceptibility to amantadine was evaluated for a total of 178 influenza A virus strains, 127 of A(H3N2) and 51 of A(H1N1) subtype, isolated from the 2004/2005 to the 2006/2007 winter seasons (Table 1). This evaluation was carried out at the Health Protection Agency (London, United Kingdom) during 2007, using all influenza A virus strains (from the winter seasons referred) for which a sufficient volume for performing the analysis was available at that moment. The majority of these strains were tested using a 2nd or 3rd cell passage isolate. A total of 340 influenza virus strains isolated from the 2004/2005 to the 2008/2009 winter seasons, 117 of A(H3N2) subtype, 93 of A(H1N1) subtype and 130 of B type, were tested for oseltamivir susceptibility by fluorescence assay (Table 1). The majority of these strains were tested using a 2nd to 4th cell passage isolate. From all the strains isolated during the winter seasons considered for this study, only those that did not exhibited a NA activity equal or higher than 25,000 relative fluorescence units (RFUs), after all the possible re-isolations procedures for increasing activity, were not tested. Wild-type reference influenza virus strains susceptible to NAIs were used as assay controls on fluorescence assay. These were: A/Wisconsin/67/2005 (A(H3N2) subtype); A/Texas/36/1991 (A(H1N1) subtype) and; B/Memphis/20/1991 (B type). These reference strains were kindly provided by Dr. Alan Hay (National Institute for Medical Research, Mill Hill, London, United Kingdom). From the 340 virus strains tested with the fluorescence assay, 134 were further analyzed by NA gene sequencing, including all strains identified as statistical outliers and approximately 25% of non-outliers (randomly selected) (Table 1).

2.2. Oseltamivir

Oseltamivir carboxylate, the active compound of the ethyl ester prodrug oseltamivir phosphate, was provided in the form of a D-tartrate salt by F. Hoffmann-La Roche Ltd. (Basel, Switzerland), through a material transfer agreement.

2.3. Susceptibility to amantadine

Susceptibility to amantadine was evaluated by pyrosequencing using the standard operating procedure (SOP) provided by the European Surveillance Network for Vigilance against Viral Resistance (VIRGIL), which included (1) the amplification of the influenza A M2 gene for amantadine sensitivity pyrosequencing and (2) the preparation of DNA for pyrosequencing using the PyroMark Vacuum Prep Workstation. The 2 steps of the VIRGIL SOP were based on the pyrosequencing manufacturer's protocol (Biotage, Uppsala, Sweden) previously described by Bright et al. (2005). Two relevant modifications were introduced in each step. For the amplification of the M2 gene, 5 μl of viral RNA was used in a 50 μl reaction mixture and the number of amplification cycles was increased to 35. For the preparation of DNA for pyrosequencing, the M2 sequencing primer was used at a final concentration of 0.44 μM and the duration of the annealing step was halved (2 min). Pyrograms were obtained through a SNP (AQ mode) analysis specific for the 5 point mutations associated with the development of resistance to amantadine: L26F/I; V27A/D; A30T; S31N; and G34E (Hay et al., 1985,

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Table 1
Number of influenza virus strains analyzed for amantadine and oseltamivir susceptibility/number of influenza virus strains isolated at national level (percentage), by influenza (sub)type or B lineage and winter season.

Winter season	A(H3N2)			A(H1N1)			B						Total[a]		
	Amantadine	Oseltamivir		Amantadine	Oseltamivir		Oseltamivir						Amantadine	Oseltamivir	
	Pyro	Fluo	NA seq	Pyro	Fluo	NA seq	Fluo			NA seq			Pyro	Fluo	NA seq
							Yam	Vic	Both	Yam	Vic	Both			
2004/2005	75/89 (84.3)	76/89 (85.4)	20/80 (22.5)	6/6 (100)	4/6 (66.7)	3/6 (50)	31/33 (93.9)	22/22 (100)	33/35 (94.3)	1/33 (3.0)	1/2 (50)	2/35 (5.7)	81/95 (85.3)	113/130 (86.9)	25/130 (19.2)
2005/2006	1/1 (100)	1/1 (100)	1/1 (100)	46/46 (97.8)	39/46 (84.8)	34/46 (304.4)	1/1 (100)	4/4 (100)	4/4 (100)	0/1 (100)	2/4 (42.2)	2/4 (44.4)	46/47 (97.9)	80/86 (92.2)	17/86 (17.7)
2006/2007	51/76 (67.1)	20/76 (263)	15/76 (19.7)	0/29 (0)[b]	0[b]	0[b]	1/1 (100)	1/1 (100)	2/2 (100)	1/1 (100)	1/1 (100)	2/2 (100)	51/76 (67.1)	22/76 (28.2)	17/78 (21.8)
2007/2008	0[b]	0[b]	0[b]	0/29 (0)[c]	29/29 (100)	22/29 (75.9)	43/43 (100)	2/2 (100)	45/45 (100)	16/45 (35.6)	1/2 (50)	17/45 (37.8)	0/29 (0)[c]	74/74 (100)	39/74 (52.7)
2008/2009	0/21 (0)[c]	20/21 (95.2)	14/21 (66.7)	0/21 (0)[c]	21/21 (100)	21/21 (100)	0[b]	1/1 (100)	1/1 (100)	0[b]	1/1 (100)	1/1 (100)	0/42 (0)[c]	42/43 (97.7)	36/43 (83.7)
Total	127/187 (67.9)	117/187 (62.6)	50/187 (26.7)	51/102 (500)	93/102 (91.2)	60/102 (58.8)	76/78 (97.4)	54/54 (100)	130/132 (985)	18/78 (23.1)	6/54 (11.1)	24/132 (18.2)	178/289 (61.6)	340/421 (80.8)	134/421 (31.8)

The abbreviations used in the table correspond to: pyrosequencing (Pyro); fluorescence assay (Fluo); and neuraminidase gene sequencing (NA seq).
[a] Considering both lineages of influenza B virus.
[b] None influenza virus strain from this subtype/B lineage was isolated during this winter season.
[c] Pyrosequencing was not performed on influenza A isolates from this winter season.

1991; Hurt et al., 2007). These procedures were carried out at the Health Protection Agency (London, United Kingdom).

2.4. Susceptibility to oseltamivir

Susceptibility to oseltamivir was first evaluated by a phenotypic fluorescence assay and then through sequencing of NA gene coding region.

2.4.1. Fluorescence assay

Fluorescence assay was performed using the SOP provided by VIRGIL for determination of influenza virus susceptibility to neuraminidase inhibitors, available to the general public from http://www.nisn.org/documents/Zambon_-_VIRGIL_IC50_SOP.pdf. This SOP is based on the method developed by Potier et al. (1979), in which the amount of fluorescent product 4-methylumbelliferone that is cleaved from the substrate 2′,2′-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid sodium salt hydrate (MUNANA) by the NA of influenza viruses is measured. A NA activity assay was initially performed in order to ensure that equivalent NA activities were compared against oseltamivir. For each influenza virus strain, the dilution in the linear portion of the enzyme activity curve to be used in the subsequent NA inhibition assay was determined. With this second assay, the concentration of oseltamivir required to inhibit 50% of the NA activity of each influenza virus strain (IC_{50} value) was determined.

2.4.2. NA gene sequencing

RNA extraction was performed either using a modified version of the protocol described by Boom et al. (1990) or the QIAamp Viral RNA Mini Kit (QIAGEN, Germany). A conventional two-step RT-PCR was used for the amplification of 3 overlapping segments, with a length varying from 490 to 700 bp, which covered the entire NA gene sequence. RT reaction was performed as described by Ellis et al. (1997) and the PCR reaction consisted of 10 μl of cDNA added to 40 μl of a reaction mixture containing 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 1 mM (N1 subtype and B type) or 1.25 mM (N2 subtype) $MgCl_2$, 12.5 pmol (N1 subtype and B type) or 5 pmol (N2 subtype) of each primer, and 1.5 U of Taq DNA polymerase. PCR cycling conditions included: 2 min at 95 °C; 35 cycles of 1 min denaturation at 94 °C, 1 min annealing at 50 °C (N1 and N2 subtypes) or 52 °C (B type) and 2 min extension at 72 °C; and cooling at 8 °C. Purification of the amplified segments was carried out using the QIAquick PCR Purification Kit (QIAGEN, Germany), and the resulting DNA quantified by spectrophotometry. Nucleotide sequences of the purified segments were obtained on an automatic sequencer ABI PRISM Biosystems 3130XL Genetic Analyser after using the Big Dye Terminator Cycle Sequencing Ready Reactions kit v1.1, both according to the manufacturer's instructions (Applied Biosystems). For each of the 3 different amplified segments, 3 sequencing primers were used (2 forward and 1 reverse). The 9 overlapping sequences of NA gene, obtained for each influenza virus strain, were then assembled and edited using the SeqMan II software from the Lasergene package v4.05 (DNASTAR Inc.). The alignment of the NA coding sequences obtained for each NA (sub)type was performed with the Megalign software (from same package) using the neighbour-joining method. NA sequences of reference influenza virus strains, obtained from Influenza Sequence Database at https://isd.eiss.org/, were included in the alignments. The sequences of all primers used are available from the authors upon request.

2.5. Statistical analysis

The temporal distribution of the notification forms indicating (1) prescription or (2) no prescription of influenza antiviral drugs,

Table 2
Frequency (number) of influenza-like illness notification forms received with the indication of prescription and of no prescription of antiviral drugs during the 4 winter seasons for which this information was available (2005/2006 to 2008/2009).

Type		Winter season				p^a
		2005/2006 (N = 298)	2006/2007 (N = 493)	2007/2008 (N = 226)	2008/2009 (N = 600)	
Prescription	% (n)	2.0 (6)	2.2 (11)	3.1 (7)	1.0 (6)	0.191
	IC95%	0.74–4.33	1.12–3.96	1.25–6.28	0.37–2.16	
No prescription	% (n)	98.0 (292)	97.8 (482)	96.9 (219)	99.0 (594)	
	IC95%	95.67–99.26	96.04–98.88	93.72–98.75	97.84–99.63	
No information	n	240	237	67	129	-

[a] Pearson's Chi-Square Test.

was analyzed by a Pearson Chi-Square Test. Additionally, 95% confidence intervals were determined according to Bliss (1967).

Regarding the fluorescence assay, NA activity and IC_{50} values were calculated through point-to-point curve fitting using the Microsoft Office Excel 2003 (Microsoft Office Professional Edition 2003). The identification of outliers was performed through the determination of lower and upper IC_{50} cut off values for each influenza (sub)type and winter season. The lower cut off value corresponded to 1.65 standard deviations (SD) and the upper cut off value to 3SD above the median IC_{50} value, using a robust estimation of the SD (AMC, 2001). Any influenza virus strain with an IC_{50} value higher than the lower cut off was classified as a minor outlier, and as a major outlier when the IC_{50} value was higher than the upper cut off. All statistical outliers identified were retested twice and the mean IC_{50} value was considered for analysis. An IC_{50} baseline level was also determined for each influenza (sub)type and winter season, corresponding to the median value ± 1 robust SD (outliers not included). This same determination (median value ± 1 robust SD) was performed for each of the 2 influenza B lineages in co-circulation (B/Yamagata and B/Victoria) but, in this case, outliers were included. The variation of IC_{50} values between winter seasons and influenza (sub)types/B lineages was analyzed performing an ANOVA one-way. If significant differences were identified, these were analyzed, if possible, by Bonferroni's correction post hoc test. All data obtained with the fluorescence assay was log-transformed before being used for statistical analysis in order to be normally distributed.

All statistical tests were performed using the SPS Statistics software v17.0 and, in all of them, a p value <0.05 was considered to be statistically significant.

3. Results

3.1. Prescription and exposure to influenza antiviral drugs

The information on antiviral drugs indicated on the notification forms received revealed that, from 2005/2006 to 2008/2009, antiviral drugs were rarely prescribed for the treatment of influenza-like illness symptoms. More specifically, indication of antiviral drug prescription was only found on 30 (0.02%) of the 1617 notification forms received during the winter seasons analysed. No significant differences were observed in the prescription frequency throughout the seasons (Pearson Chi-Square Test, p = 0.191) (Table 2).

Of the 30 cases of antiviral drug prescription notified, 27 (90.0%) were received through the Emergency Units Network of the National Influenza Surveillance Programme (Table 3). No particular pattern was observed when analysing the distribution of prescription cases during each winter season and by geographic origin (cases were from 5 regions of mainland Portugal). The same was not observed when considering the patient age group, with the majority of antiviral prescriptions occurring in patients belonging to the 15–44 years age group (22; 73.3%), which includes adolescents and younger adults. Of the 30 notifications of antiviral drug prescription, 4 (13.3%) were for patients who had previously been vaccinated for influenza. Two of these notifications, the 1 from 2005/2006 and the first of 2006/2007, occurred at the beginning of the season and originated on 2 young adults, while the other 2 occurred during the epidemic period of the season and were from an adult and from an older person (Table 3). Oseltamivir was the drug most prescribed, having been indicated in 19 (90.5%) of the 21 known antiviral prescriptions. Laboratory analysis indicated that influenza viruses were detected in only approximately half of the nasopharyngeal swabs associated with antiviral prescription (14; 46.7%). However, it is important to notice that in the last season (2008/2009) there was an absolute association between antiviral prescription and positive influenza infection (Table 3). The second respiratory specimen was obtained only from the 2 influenza positive cases detected in 2007/2008 and from 3 of the 6 ones detected in 2008/2009. Isolation, however, was unsuccessful.

Regarding the situation of exposure of patients to antiviral drugs before specimen collection, only 3 cases were notified between 2005/2006 and 2008/2009 (1 in 2006/2007 and 2 in 2008/2009). However, the antiviral to which the patients were exposed was not referred and the respiratory specimens that came with these notifications were negative for influenza virus infection. Because of this and of the lack of success on viral isolations performed on the second respiratory specimens, it was not possible to analyse the impact of antiviral drug use on influenza virus susceptibility to oseltamivir and amantadine.

3.2. Susceptibility to amantadine

Amantadine resistant marker S31N was identified on the M2 protein sequence of 39 (30.7%) of the 127 influenza A(H3N2) virus strains analyzed, specifically on the single virus strain from 2005/2006 and on 38 (74.5%) of the 51 virus strains from 2006/2007. In the origin of this resistance is a G to A substitution in the second nucleotide of the codon that codes for the amino acid at position 31 of the M2 protein sequence. Using the epidemiological information available, it was possible to observe that A(H3N2) amantadine resistant strains from 2006/2007 were isolated from respiratory specimens collected during the whole winter season and from patients of all age groups and from almost all geographic regions of mainland Portugal.

Regarding the A(H1N1) subtype, all 51 influenza virus strains analysed were found to be susceptible to amantadine, since none of the 5 molecular markers of resistance to this antiviral drug was identified in their M2 protein sequence.

3.3. Susceptibility to oseltamivir

3.3.1. Fluorescence assay

3.3.1.1. IC_{50} baseline level. No significant differences were observed on the IC_{50} baseline levels obtained for the influenza A(H3N2) subtype (ANOVA one-way, p = 0.303) (Table 4). This situation was not observed, however, for both influenza A(H1N1)

Table 3
Epidemiological and laboratory information regarding each of the 30 cases of antiviral drug prescription notified during the 4 winter seasons for which this information was available (2005/2006 to 2008/2009).

Winter season	Week	Notification form						1st nasopharyngeal swab		2nd nasopharyngeal swab	
		Source	Geographic origin	Patient			Antiviral	Infection with influenza virus	Isolation in cell culture	Infection with influenza virus	Isolation in cell culture
				Gender	Age (years)[a]	Vaccination					
2005/2006	41	EU	Alentejo	F	54	No	NR	N	–	–	
	44	EU	North	F	69	No	NR	N	–	–	–
	45	EU	Lisboa	F	43	Yes	NR	P (B type)	–	–	–
	48	EU	Alentejo	F	22	No	NR	N	–	–	
	2	EU	North	F	NR	No	NR	N	–	–	
	3	EU	North	M	34	NR	NR	N	–	–	–
2006/2007	44	EU	Center	M	23	No	Oseltamivir	N	–	–	–
	50	EU	Center	F	31	Yes	NR	N	–	–	–
	2	EU	Alentejo	F	29	No	Oseltamivir	N	–	–	
		EU	Alentejo	F	44	No	Oseltamivir	P (subtype AH3)	–	–	
		EU	Alentejo	M	27	No	Oseltamivir	P (subtype AH3)	–	–	–
	6	SMP	Center	M	48	Yes	Oseltamivir	N	–	–	–
		SMP	Alentejo	F	NR	No	NR	N	–	–	–
		EU	Algarve	M	50	No	Oseltamivir	P (subtype AH3)	–	–	
	7	EU	Lisboa	M	28	No	Oseltamivir	P (subtype AH3)	–	–	
	8	EU	Alentejo	M	23	No	Oseltamivir	P (subtype AH3)	–	–	–
		EU	Alentejo	M	39	No	Oseltamivir	N	–	–	–
2007/2008	47	–	Lisboa	M	41	No	NR	N	–	–	–
	51	EU	Alentejo	M	22	No	Oseltamivir	N	–	–	–
		EU	Center	M	29	No	Oseltamivir	P (B type)	P (B/Lisboa/12/2008)	P (B type)	N
2008/2009	51	EU	North	F	34	No	Zanamivir	P (subtype AH3)	P (B/Lisboa/45/2008)	N	–
	1	SMP	Algarve	F	73	Yes	Oseltamivir	P (subtype AH3)	P (A/Lisboa/45/2008)	P (A type)	N
	3	EU	North	F	26	No	Oseltamivir	P (subtype AH 1)	P (A/Lisboa/19/2009)	–	
	6	EU	Center	F	29	No	Oseltamivir	P (subtype AH1)	–	N	
	7	EU	Center	M	20	No	Oseltamivir	P (subtype AH1)	P (A/Lisboa/23/2009)	–	–
	9	EU	Center	F	14	No	Oseltamivir	P (subtype AH 1)	P (A/Lisboa/21/2008)	–	–

The abbreviations used in the table correspond to: Emergency Units (EU); Sentinel Medical Practitioners (SMP); male (M); female (F); not referred (NR); negative (N); positive (P).
[a] The following age group distribution was considered in the analysis: 0–4 younger children; 5–14 children and younger adolescents; 15–44 adolescents and younger adults; 45–64 adults; 65 or plus elderly.

Table 4
Oseltamivir IC_{50} baseline levels for each influenza (sub)type and winter season.

Influenza (sub)type	Median ± robust SD					p[a]	
	2004/2005	2005/2006	2006/2007	2007/2008	2008/2009	Between seasons	Between (sub)types
A(H3N2)	0.38 ± 0.13	0.24[b]	0.41 ± 0.04	–[c]	0.38 ± 0.10	0.303	
A(H1N1)	1.71 ± 0.11	1.47 ± 0.49	–[c]	1.88 ± 0.39	526.35 ± 201.75	0.000 (0.044 without 08/09)	0.000
B	13.08 ± 3.47	21.04 ± 3.01	18.82 ± 6.86[d]	19.59 ± 7.09	38.41[b,e]	0.000	

[a] ANOVA one-way.
[b] Only 1 influenza virus strain from this subtype was isolated during this winter season.
[c] None influenza virus strain from this subtype was isolated during this winter season.
[d] Only 2 influenza virus strains from this subtype were isolated during this winter season.
[e] Minor outlier but is the only B IC_{50} value obtained in this winter season.

Table 5
Oseltamivir IC_{50} baseline levels for each influenza B HA lineage and winter season.

Influenza B lineage	Median ± robust SD					p[a]	
	2004/2005	2005/2006	2006/2007	2007/2008	2008/2009	Between seasons	Between (sub)types
Yamagata	13.06 ± 3.13	14.35[b]	14.19[b]	20.12 ± 7.12	–[c]	0.000	
Victoria	21.74 ± 3.97[d]	21.27 ± 3.14	23.45[b]	16.20 ± 0.81[d]	38.41[b]	0.001	0.000
p[a]	0.005	0.011	–	0.254	–	–	

[a] ANOVA one-way.
[b] Only 1 influenza B virus from this lineage was isolated during this winter season.
[c] None influenza B virus from this lineage was isolated during this winter season.
[d] Only 2 influenza B virus from this lineage were isolated during this winter season.

subtype and type B viruses. The IC_{50} baseline levels determined for the A(H1N1) subtype differed markedly between the first 4 winter seasons analysed and the 2008/2009 season (ANOVA one-way, $p < 0.05$, Bonferroni's correction post hoc test). This high difference of approximately 300-fold, indicates a potential shift on the antiviral drug profile of A(H1N1) virus, from susceptible to resistant, between 2007/2008 and 2008/2009. Additionally, when removing the 2008/2009 IC_{50} values from the analysis, A(H1N1) IC_{50} baseline levels of 2005/2006 and 2007/2008 proved to be significantly different (ANOVA one-way, $p < 0.05$, Bonferroni's correction post hoc test). Regarding influenza type B, IC_{50} baseline levels varied significantly throughout winter seasons (ANOVA one-way, $p < 0.05$). However, it was not possible to perform the analysis of these differences through a post hoc test due to the existence of a single value for 2008/2009 (Table 4).

Fig. 1. IC_{50} values obtained by fluorescence assay for the influenza A(H1N1) (a), the influenza A(H3N2) (b) and the influenza B virus strains analyzed (c), in each of the winter seasons studied. Each (♦), (◊) and (◆) represents an IC_{50} value obtained, being that the last 2 symbols represent also the minor and major outliers identified, respectively. The (·····) represents the IC_{50} median value, the (ssssss) the lower IC_{50} cut off value and the (_____) the upper IC_{50} cut off value. *Note*: Lower and upper cut off values were only determined when the number of virus strains tested were equal or higher than 10. When this did not occur, we considered for analysis the cut off values of the previous and/or the following winter season.

Oseltamivir IC_{50} baseline levels proved to differ significantly according to the NA (sub)type of influenza viruses, with NA type B virus exhibiting the higher levels, followed by the NA subtype A(N1) and by the NA subtype A(N2) virus (ANOVA one-way, $p < 0.05$) (Table 4).

Additionally, oseltamivir IC_{50} baseline levels differed significantly between the 2 co-circulating influenza B HA lineages, B/Yamagata and B/Victoria (ANOVA one-way, $p < 0.05$) (Table 5). B/Yamagata-like virus from 2004/2005 and 2005/2006 showed significant lower IC_{50} baseline levels when compared to B/Victoria-like virus from the same winter seasons (ANOVA one-way, $p = 0.005/p = 0.011$). For the remaining 3 seasons, the difference observed was not statistically significant, or it was not possible to perform the analysis. Significant differences were also observed on the IC_{50} baseline levels obtained for each influenza B lineage throughout the winter seasons (ANOVA one-way, $p < 0.05$). These differences appeared to occur between the 2007/2008 and the previous 3 winter seasons for the B/Yamagata lineage and between the 2008/2009 and the previous 4 winter seasons for the B/Victoria lineage (Table 5). However, the impossibility of performing a post hoc test does not allow confirmation at statistical level.

3.3.1.2. IC_{50} values and outlier determination. Regarding A(H1N1) influenza subtype, IC_{50} values ranged from 1.63 to 2.13 nM in 2004/2005, from 0.83 to 2.6 nM in 2005/2006, from 0.8 to 2.6 nM in 2007/2008 and from 314.04 to 1208.84 nM in 2008/2009 (Fig. 1a). All A(H1N1) virus strains from 2008/2009 were directly considered as potential oseltamivir-resistant strains, as a result of the high IC_{50} values exhibited by all of them. During the previous 4 winter seasons, 2 minor and 6 major A(H1N1) outliers were identified by statistical analysis. The virus strains classified as minor outliers exhibited an approximately 2-fold decrease in the susceptibility to oseltamivir (based on the IC_{50} median value). These were: A/Lisboa/25/2006 (IC_{50} of 2.85 nM), from 2005/2006; and A/Lisboa/8/2008 (IC_{50} of 3.02 nM), from 2007/2008. The 6 major outliers identified in 2007/2008 were: A/Lisboa/2/2008 (IC_{50} of 609.09 nM); A/Lisboa/3/2008 (IC_{50} of 584.82 nM); A/Lisboa/11/2008 (IC_{50} of 441.90 nM); A/Lisboa/20/2008 (IC_{50} of 1064.15 nM); A/Lisboa/27/2008 (IC_{50} of 642.05 nM), and; A/Lisboa/28/2008 (IC_{50} of 511.44 nM). These virus strains exhibited a 230–560-fold decrease in susceptibility to oseltamivir (based on the IC_{50} median value), having been, therefore, considered as potential oseltamivir-resistant strains (Fig. 1a).

Influenza A(H3N2) IC_{50} values ranged from 0.18 to 0.61 nM in 2004/2005, from 0.25 to 0.47 nM in 2006/2007 and from 0.28 to 0.54 nM in 2008/2009 (Fig. 1b). Only 2 A(H3N2) outliers were identified by statistical analysis, 1 minor and 1 major. These 2 outliers were from the 2006/2007 winter season, corresponding to the virus strains A/Lisboa/1/2007 (IC_{50} of 0.52 nM—minor) and A/Lisboa/54/2007 (IC_{50} of 0.6nM—major) (Fig. 1b). However, the difference observed between their IC_{50} values and the seasonal IC_{50} median value was less than 2-fold.

For influenza type B, IC_{50} values ranged from 7.73 to 19.39 nM in 2004/2005, from 14.35 to 26.72 nM in 2005/2006 and from 11.90 to 33.61 nM in 2007/2008 (Fig. 1c). A total of 4 minor outliers were identified, corresponding to the virus strains: B/Lisboa/12/2005 (IC_{50} of 24.41 nM), from 2004/2005; B/Lisboa/5/2006 (IC_{50} of 30.51 nM) and B/Lisboa/11/2006 (IC_{50} of 27.53 nM), from 2005/2006, and; B/Lisboa/1/2009 (IC_{50} of 38.41 nM), from 2008/2009 (Fig. 1c). Both B/Lisboa/12/2005 and B/Lisboa/1/2009 outlier strains exhibited an approximately 2-fold decrease in the susceptibility to oseltamivir (based on the IC_{50} median value), while for the other 2 outlier strains the difference observed was less than 2-fold.

3.3.2. NA gene sequencing

The analysis of the A(H1N1) virus NA sequences revealed the presence of the mutation His275Tyr (H275Y, N1 numbering) in the sequence of the 6 virus strains from 2007/2008 classified as major outliers on the fluorescence assay, and in all virus strains from 2008/2009. This mutation is associated with a high level of resistance to oseltamivir in the NA subtype (N1) virus, resulting from a C to T nucleotide substitution at position 823 of the coding sequence. Its identification in the NA sequence of the A(H1N1) virus strains that exhibited high fluorescence IC_{50} values allowed to confirm that 6 (20.7%) of the 29 A(H1N1) virus strains from 2007/2008 and that all (100%) A(H1N1) virus strains from 2008/2009 were resistant to oseltamivir. The NA sequence of these oseltamivir-resistant viruses is also characterized by the presence of the mutation Asp354Gly (D354G, N1 numbering), in relation to the reference strain A/Brisbane/59/2007. No amino acid changes different from those observed in the other co-circulating A(H1N1) virus strains were observed in the NA sequence of the 2 strains classified as minor outliers on the fluorescence assay.

The lack of different amino acid changes that could explain the reduction observed for oseltamivir susceptibility was also verified in the NA sequence of the 2 A(H3N2) virus strains classified as outliers on the fluorescence assay. However, the presence of a mixed population of virus with either an Asp (D) or an Asn (N) at residue 151 (G and A peak at position 451 of the N2 coding sequence) was found in the NA sequence of the non-outlier A/Lisboa/8/2007 virus strain, from 2006/2007. The mutation D151N has been associated with a reduction in the susceptibility of influenza A and B viruses to oseltamivir and/or zanamivir. However, this virus strain exhibited one of the lowest IC_{50} values (0.29 nM) obtained during 2006/2007.

Regarding influenza type B, the NA sequences of the 4 virus strains classified as minor outliers in the fluorescence assay were analysed in relation to NA sequences of reference B virus strains, and not to NA sequences of co-circulating B virus strains as performed for A(H1N1) and A(H3N2) subtypes. This difference is a consequence of the low number of influenza B strains isolated during 2006/2007 and 2008/2009 and of the few influenza B strains from 2004/2005 that were sequenced for NA. Two mutations were found in the NA sequence of the outlier B/Lisboa/12/2005: Ile240Val (I240V) and Glu404Gly (E404G) (B numbering). In the NA sequence of the outlier B/Lisboa/1/2009, 2 mutations were also identified: Arg65Leu (R65L) and Ala358Glu (A358E). None of these 4 mutations are associated with reduction or with development of resistance to oseltamivir. In the NA sequence of the 2 outliers from 2005/2006, B/Lisboa/5/2006 and B/Lisboa/11/2006, no different amino acid changes were observed that could explain the reduced susceptibility.

4. Discussion

This study revealed that antiviral drugs were rarely prescribed at national level for the treatment of seasonal influenza, from 2005/2006 to 2008/2009. This may be a direct consequence of the perception that these antivirals would be crucial and should only be used during a pandemic. The fact that the information obtained is limited to the population under observation, in the context of the National Influenza Surveillance Programme, should also be taken into account. In fact, the information was collected from patients that use primary and secondary public health care systems where the delay in consultation upon onset of symptoms leads to unsuitability of antiviral drug prescription. Most of the antiviral prescriptions were indicated for patients belonging to the 15–44 age group (adolescents and younger adults), but no information was available about their risk of developing serious complications from influenza. This information could have been useful when analyzing the 4 cases of drug prescription in previously vaccinated

individuals that, most probably due to recent vaccination, did not have a sufficient level of immunity against circulating influenza viruses. Oseltamivir was the antiviral drug most prescribed. The reasons for this could have been its advantage over zanamivir in terms of administration route and pharmacokinetics, and the high level of resistance observed for M2 inhibitors (Democratis et al., 2006; Deyde et al., 2007). Additionally, M2 inhibitors in Portugal are essentially used for treatment of Parkinson's disease. One important finding of this study, which raises concerns on how antiviral drugs are being used, was that approximately half of the prescriptions were made for individuals who were not infected with influenza.

Resistance to amantadine was only observed for the A(H3N2) influenza subtype, with the identification of the mutation S31N on the M2 protein sequence of the only strain isolated in 2005/2006 and of 74.5% of the strains from 2006/2007. This mutation is the most common mechanism of resistance to M2 inhibitors, being already known that is occurrence on the M2 protein constricts the size and increases the polarity of the antiviral drug binding site (Stouffer et al., 2008; Weinstock and Zuccotti, 2006). The situation observed at the national level is in agreement with the global spread of an A(H3N2) virus strain carrying the S31N mutation that has been observed since 2002/2003 (Bright et al., 2005).

In respect to oseltamivir, resistance was only observed for seasonal A(H1N1) influenza virus and at an increasing frequency of 20.7% in 2007/2008 to 100% in 2008/2009. These resistant viruses exhibited extremely high IC_{50} values, between 314.04 and 1208.84 nM, and carried the mutation H275Y (N1 numbering) in their NA protein sequence. The mechanism of resistance associated with the presence of this mutation is already known, consisting of prevention of formation of the pocket in the NA active site of influenza N1 subtype virus, which is essential for the high affinity binding of oseltamivir (Moscona, 2005; Wang et al., 2002). The role of the D354G mutation that was also found in the NA sequence of all A(H1N1) oseltamivir-resistant virus is still unclear. The situation observed at the national level is in agreement with the widespread and sustained transmission of oseltamivir A(H1N1) resistant virus observed worldwide since 2007/2008 (ECDC, 2008). Until now, no clear reasons were found for the emergence and global spread of this A(H1N1) resistant virus.

No different mutations, comparing to those observed in other co-circulating strains from the same subtype, were identified in the NA sequence of the A(H1N1) and A(H3N2) virus strains that exhibited a reduction for oseltamivir susceptibility at the phenotypic level (fluorescence minor outliers). For this reason, a more detailed genotypic analysis of these strains will be attempted, involving the sequence of other segments of the genome, particularly HA. The presence of the mixed population of D151 wild-type and N151 mutant virus on the non-outlier A/Lisboa/8/2007 virus strain is now being further studied, given the described association of D151N mutation to a reduction on NAIs susceptibility (McKimm-Breschkin et al., 2003; Sheu et al., 2008). For influenza type B, due to the limited number of sequences available, it was not possible to verify if the 2 mutations identified exclusively on 2 of the minor outliers (B/Lisboa/12/2005 and B/Lisboa/1/2009) were associated with the reduction on oseltamivir resistance, observed at phenotypic level, or are simply a result of antigenic drift. However, it has to be taken into consideration that (1) B/Lisboa/12/2005 belongs to the B/Victoria lineage and was isolated during a season of B/Yamagata lineage dominance and; (2) that B/Lisboa/1/2009 was the only B virus isolated during 2008/2009, a season in which the occurrence of a HA and NA antigenic drift on the B/Victoria lineage, from B/Malaysia/2506/2004 to B/Brisbane/60/2008-like virus, was described. Further genotypic analysis of influenza B virus, involving NA sequencing and sequencing of other segments of the genome of outlier virus strains would be essential.

Susceptibility of influenza viruses to oseltamivir proved to differ significantly according to the NA (sub)type. This difference was described in previous studies and was expected to occur here, having been associated with the existence of minor structural differences between the NA active sites of each influenza NA sub(type) virus, which would result in different oseltamivir binding affinities (Boivin and Goyette, 2002; Escuret et al., 2008; Ferraris et al., 2005; Hurt et al., 2004; McKimm-Breschkin et al., 2003). The reduction in susceptibility to oseltamivir observed from 2005/2006 to 2007/2008 in the A(H1N1) subtype was most probably a result of NA antigenic drift, from A/New Caledonia/20/1999-like virus to A/Brisbane/59/2007-like virus, that occurred between these winter seasons (results not shown). The co-circulation of 2 HA B lineages (B/Yamagata and B/Victoria) could help to explain the variation observed in the susceptibility of influenza B virus during the period of this study. These 2 lineages differed significantly in their susceptibility to oseltamivir, with B/Yamagata-like virus exhibiting significant lower IC_{50} values for 2 of the 3 winter seasons for which this analysis was possible to be performed. Additionally, this difference between B lineages has already been described by Lackenby et al. (2008b).

For the coming winter season(s), it would be of great importance to continue these studies and to advance to more specific research studies, even more now that the importance and critical role of antiviral drugs, particularly oseltamivir, has been demonstrated and highlighted for the A(H1N1) 2009 influenza pandemic.

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